Universidade do Minho
Escola de Engenharia

Cátia Sofia Almeida Cardoso

Improvement of oral health: biofilm formation, oral implants and probiotics

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Dissertação de Mestrado
Ciclo de Estudos Integrados Conducentes
ao Grau de Mestre em Engenharia Biomédica

Trabalho realizado sob a orientação da
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e do
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Abstract

Oral health problems affect a large part of the world population and despite the numerous developments on technologies and products, there is still a need to know and understand those diseases. The recurring demands from society led to the development of new medical treatments and new materials used in dental implants. So, in this context, it became very important to evaluate the microbial colonization of implant materials because these materials can be important for future dental applications being necessary know the microbial adhesion. Therefore, the present dissertation aimed to improve the knowledge on oral microbial colonization of oral cavity.

The first goal was the evaluation of microbial colonization of different titanium surfaces (anodized and etched) that are normally used in implant applications. The bacteria studied (*Prevotella intermedia, Porphyromonas gingivalis*, *Fusobacterium nucleatum*) were able to form biofilms on both surfaces, although biofilm formation on anodized samples result in a higher amount of biomass than in the etched samples, although with a similar number of viable cells, indicating the higher presence of extracelular matrix in the former, which could induce lower mechanical friction on these samples.

In addition this study also evaluated the influence of fluoride and probiotic bacteria (*Streptococcus salivarius*) on biofilm formation. The presence of fluoride showed to inhibit biofilm formation on these biomaterials. The effect of probiotic bacteria has been evaluated on biofilm formation and *S. salivarius* had a direct influence on reducing the growth of pathogenic bacteria, such as *F. nucleatum*. However, these interactions are still unclear and there is a need to study these in greater detail.

Moreover, dental implants infection can also be reduced by controlling the presence of pathogenic species in oral environment, so another aim was the evaluation of the use of a sugar (C7) as a prebiotic agent. The different sources of energy (C7 sugar and glucose) had different influences on growth of pathogenic and probiotic bacteria. So, the sugar, C7, can be used to favour the growth of the beneficial oral bacteria.

In conclusion it can be pointed out that Ti anodized samples may be a good material for the production of dental implants due to their topography and also that the use of different energy sources allied with probiotics may be a start point for the development of new therapies.

Resumo

Os problemas de saúde oral afectam grande parte da população mundial e apesar do grande desenvolvimento nas tecnologias e nos produtos aplicados a esta, existe ainda a necessidade de conhecer e compreender melhor as doenças orais de origem bacteriana. Assim, a presente dissertação teve como objectivo geral contribuir para melhorar o conhecimento sobre a interação bacteriana na cavidade oral.

O primeiro objectivo deste trabalho foi a avaliar a formação de biofilme em diferentes superfícies de titânio (anodizadas e com tratamento químico) normalmente utlizadas em implantes dentários. As bactérias estudadas (*Prevotella intermedia, Porphyromonas gingivalis*, *Fusobacterium nucleatum*) foram capazes de formar biofilme em ambas as superfícies, embora nas amostras anodizadas a quantidade de biomassa formada tenha sido maior que nas com tratamento quimico. No entanto, o número de células viáveis nos biofilmes formados em ambas as superfícies foi semelhante, indicando maior presença de matriz nas amostras *anodizadas*, o que pode induzir menor fricção nestas amostras.

Além deste estudo foi também avaliada a influência da adição flúor e a presença de uma bactéria probiotica (*Streptococcus salivarius*) nos biofilmes formados nas mesma amostras. A presença de flúor no meio de cultura mostrou ter uma influencia negativa na formação de biofilme. Quanto à presença de bactéria probiotica esta foi avaliada em relação a formação de biofilme pelas mesmas bactérias patogénicas. A bactéria probiotica teve influência direta na redução da proliferação das bactérias patogénicas em especifico da *F. nucleatum*.

As infecções orais podem também ser controladas alterando o equilíbrio entre a flora patogénica e probiótica. Assim sendo, foi também avaliada a influência de um açúcar (C7) neste equilíbrio, funcionado este como um agente prebiótico. As diferentes fontes de energia (glucose - controlo - e C7), tiveram diferentes influências sobre o crescimento das bactérias patogénicas e probioticas. A fonte energia, C7, poderá ser assim usada para favorecer o crescimento da bactéria probiotica e assim contribuir para uma melhoria da saúde oral.

Portanto, neste trabalho concluiu-se que as amostras de Ti anodizado poderão ter maior potencial na produção de implantes dentários devido à sua topografia e além disso o uso de diferentes fontes de energia aliada ao uso de bactérias probióticas pode ser um início de um desenvolvimento de uma nova terapia.

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Abbreviations

BAKV:	Blood Agar plates enriched Kanamycin Vancomycin
BAP:	Blood Agar Plate for selective grow for *P. intermedia*
BHI:	Brain Heart Infusion
CFU:	Colony-Forming Unit
CV:	Crystal violet
CVE:	Crystal Violet Erythromycin agar plates
DNA:	Deoxyribonucleic acid
FN:	*Fusobacterium nucleatum*
OD:	Optical Density
PAA:	Phenylethyl Alcohol Agar
PBS:	Phosphate Buffered Saline
PI:	*Prevotella intermedia*
PG:	*Porphyromonas gingivalis*
QPCR:	Quantitative polymerase chain reaction
SEM:	Scanning Electron Microscopy
SS:	*Streptococcus salivarius*
Ti:	Titanium

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1. Chapter I

General introduction

1.1. Motivation and mains objectives

Nowadays, oral health problems affect a large part of the world population (1). Oral diseases, such as dental caries and periodontal diseases are considered a major problem in our society. These diseases are the most common bacterial diseases occurring in man and greatly contribute towards the decrease oral health. Despite the numerous developments of oral products and technologies, there is still a need to know and understand how these bacterial diseases affect general health.

The use of dental implants has been increasing exponentially over the last few decades, making it essential to understand how the bacteria react and grow in these implants. Biofilm formation on oral implants can cause inflammation of peri-implant tissues (peri-implantitis), which endangers the long-term success of osseo-integrated implants. Other oral pathologies, such as dental caries and periodontal diseases, are also related to these biofilms. Therefore, the first goal of this work was to evaluate two different types of Titanium surfaces in microbial colonization, namely biofilm formation. It was also assessed the influence of the presence of fluoride and probiotic bacteria on biofilm formation.

Moreover, the influence of a specific sugar on the growth of probiotics and pathogenic bacteria on dental implants infection can also be reduced by controlling the presence of pathogenic species in oral environment, so, another aim was the evaluation of the use of a sugar (C7) as a prebiotic agent.

Therefore, the specific aims of this work were to:

- Study the biofilm formation on titanium surfaces (anodized and etched);
- Evaluate biofilm formation in the presence of fluoride;
- Analyse biofilm formation by pathogenic bacteria in the presence of a probiotic bacteria;
- Test the effect of surface topography on biofilm formation;
- Study of the effect of different sugars (Glucose and C7) on the growth of several oral bacteria.

This study intends to complement the general knowledge about oral health in specific in biofilm formation, oral implants and probiotics.

1.2. Introduction

Numerous oral pathologies, such as dental caries and periodontal diseases are plaque-related. These diseases are probably the most common bacterial diseases occurring in man and greatly contribute towards the decrease oral health. Dental caries and periodontal disease have historically been considered some of the most important global oral health burdens.

In the case of dental caries, this is still a major health problem in most industrialized countries affecting 60–90% of school-aged children and the vast majority of adults. Poor oral health may have a profound effect on general health. The experience of pain, problems with eating, chewing, smiling and communication due to missing, discoloured or damaged teeth have a major impact on people's daily lives and well being. Furthermore, oral diseases restrict activities at home, at school and at work, causing millions of school and work hours to be lost each year throughout the world (2) (1).

Dental caries is considered a destructive condition of the dental hard tissues (teeth) that can progress to inflammation and death of vital pulp tissue, with eventual spread of infection to the periapical area of the tooth and beyond. This condition, if not treated, can lead to periodontal diseases and may even, in extreme cases, lead to tooth loss (3) (4).

Periodontal diseases are initiated by components of the plaque that develops on the tooth surface adjacent to the soft tissues of the supporting periodontium and may be confined to the gingiva (gingivitis) or extend to the deeper supporting structures with destruction of the periodontal ligament and the alveolar bone that supports the teeth (periodontitis). Periodontitis infections are characterized by the increased destruction of the periodontal ligament associated with detachment of collagen and consequent deepening of the pockets formed between the infected tissue and the teeth. Such infections associated with periodontal pocket formation, may ultimately lead to loosening and loss of the affected teeth (5). Depending on the immune response of the susceptible host, the presence of pathogenic bacterial species and the absence of beneficial bacteria, periodontitis can develop with more or less severity (6) (7).

Gingivitis, the most common form of gingival inflammation is a reversible inflammatory reaction of the dentogingival tissues (3). In contrast to gingivitis, periodontitis is greatly part a chronic inflammatory reaction of the same oral area but involving not only superficial gingival tissues but also periodontal ligament and the alveolar bone (Figure 1).

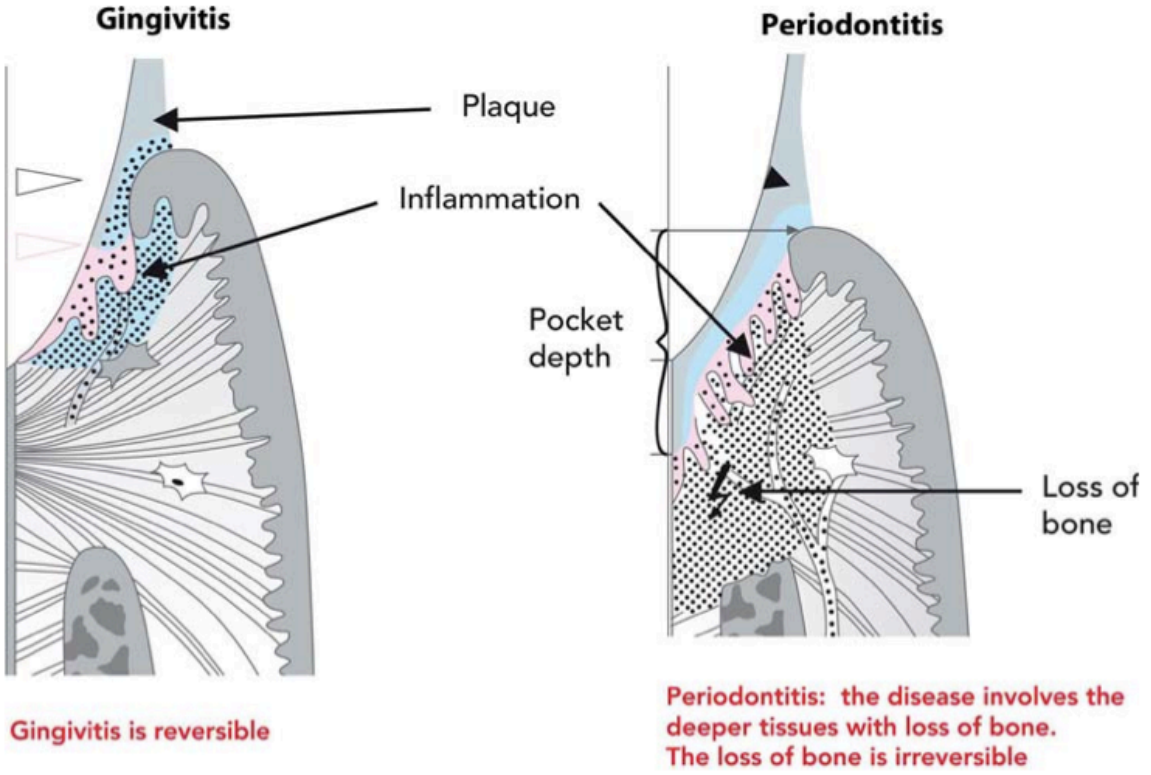

Figure 1. Representation of a chronic inflammatory infiltrate below the gingival margin (Gingivitis) and a chronic inflammatory condition affecting deeper periodontal tissues (connective tissue attachment and bone), a Periodontitis **(3)**.

The most common reported symptoms of these diseases are gingival bleeding and swelling. Other symptoms like gingival recession, drifting of teeth, mobility, and suppuration are also associated with periodontal diseases. These are signs of an advanced form of periodontitis due to progressive destruction of the dental supporting tissues. If left untreated, periodontitis results in a progressive deepening of the gingival sulcus associated to alveolar bone destruction up to the apex of the tooth, which eventually ends with its loss (8) (3) (9).

Moreover periodontitis is usually associated to polymicrobial infections of oral tissues, that result in chronic inflammation of the gingiva and surrounding connective tissue, in response to accumulations of bacteria on teeth (10).

The development of destructive periodontitis seems to be the result of a specific infection that normally starts with the formation of biofilms by specific microorganisms on tooth surfaces. The onset of these diseases is usually delayed for prolonged periods of time after initial colonization by the pathogen(s). The course of these diseases typically runs for years.

The responsible agents for colonization of oral areas, in most instances, appear to be members of the indigenous microbiota and, thus, the infections might be thought of as endogenous (3). Dental plaque biofilm formed by these endogenous bacteria on the tooth surface causes an immune response leading to the destruction of host tissues (11).

The difference between oral diseases, such gingivitis and periodontitis, and dental caries is that dental caries occurs supragingivally (on teeth above the gum line) and periodontal disease occurs subgingivally (below the gum line), attacking the tooth supporting tissues (12).

The microorganisms responsible for these disorders exhibit unique properties, conferred by their site of colonization and the nature of the environment in which they reside. They are capable to survive and to expand to other parts of the body. The microorganisms once in the blood system persist for long periods of time and they are able to adapt easily in a new environment and have high influence in other dangerous diseases (5).

1.2.1. Periodontal microorganisms

The knowledge of the complex interactions between the resident microbial communities and the human host is of extreme importance to understand the development and pathogenesis of a variety of diseases, not just the typical infectious diseases.

In the healthy oral cavity, the bacterial flora is different from that of diseased oral cavities and, often, certain indigenous bacterial species and their products are useful for a healthy periodontium (13). The commensal oral microbiota is a component of a complex homeostasis mechanism that interferes with the activity of pathogenic microorganisms (14). Commensal bacteria can affect the pathogenic species through different mechanisms, modifying the disease process by occupying a niche that could otherwise be colonized by pathogens, by actively limiting the capacity of pathogen to adhere to tissue surfaces, by affecting in a negative way the vitality or growth of a pathogen, by influencing the ability of a pathogen to produce virulence factors, or by degrading them (9). Additionally, beneficial bacteria are skilled to supply essential nutrients, regulate epithelial development, and contribute to the maturation and maintenance of the immune system (14). In other hand there are the periodontal pathogens that are the causative agents of

several periodontal diseases. For over 100 years, periodontal microbiologists have been searching for the causative agents of periodontal diseases (5).

Research indicates that there are several important species involved in the disease process, such as *Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Prevotella nigrescens, Campylobacter rectus, Capnocytophoga* spp, *Peptostreptococcus micros, Eikenella corrodens* and several species of oral spirochaetes (15) (16) (17). *Actinobacillus actinomycetem comitans*, *Porphyromonas gingivalis* and *Bacteroides forsythus*, were also strongly associated with periodontal disease status (11). These bacterial species behave in a cooperative or synergistic fashion to initiate periodontitis, a mixed anaerobic infection (18). Although pathogenic bacteria are the main factor in the aetiology of periodontitis, tissue damage is also a consequence of the host response.

Periodontal pathogens have several virulence factors that allow bacteria to colonize the host and replicate, to avoid destruction or neutralization by the defence system of the host, and to finally cause tissue damage. During an infection, important virulence factors such as adhesins, lipopolysaccharides, hemolysins, proteinases and outer membrane vesicles may act alone or in combination (18). The microorganisms can cause disease directly, by invasion of the tissues, or indirectly through production of bacterial enzymes and toxins.

Besides commensal and pathogenic oral bacteria there are an important role represented by probiotic bacteria in oral health. The term probiotic is a relatively new word meaning “for life” and is presently defined as living microorganisms, principally bacteria that are safe for human consumption and, when ingested in sufficient quantities, have beneficial effects on human health (19) (20) (21). So, in the oral cavity you have pathogenic, commensal and beneficial species, probiotics can be indigenous beneficial species or can be beneficial species that are not present in the oral cavity like commensal. Probiotics have already been successfully used to control gastro-intestinal diseases, some systemic diseases, infectious diseases such as acute diarrhea and Crohn diseases and appear to performance through colonisation resistance and/or immune modulation (19) (21) (22) (23). The oral administration of probiotics has also been explored in the control of periodontal disease (24).

Given the widespread emergence of bacterial resistance to antibiotics, the concept of probiotic therapy has been considered for application in oral health. Dental caries, periodontal disease and halitosis are among the oral disorders that have been targeted. An essential condition

for a microorganism to represent a probiotic of interest for oral health is its capacity to adhere to and colonize various surfaces of the oral cavity.

The complex environment of the oral cavity that varies for each patient, and the nutrients present in their diet are important factors to be considered. The different types of sugars present in daily diet can also influence the growth of different bacteria. The possibility to control the amount of probiotic bacteria in oral environments using the different nutrients may provide beneficial effects in oral health. The human body lives in a highly contaminated bacterial environment, and symbiosis with these microorganisms seems to be a circumstance for survival (25). Although the recent availability and widespread use of effective and cheap antibiotics has encouraged the treatment of many diseases and reduction of the death rate in many countries, it has also led to the development of resistance to a range of antibiotics (21). As well as pharmacological therapy, probiotics may be a useful adjunct to conventional therapy, not a completely alternative.

Mechanisms of probiotic action within the oral cavity can possibly be suggested from gastrointestinal studies whereby the introduction of microorganisms as a therapeutic tool for the prevention and treatment of dental caries and periodontal disease could possibly act in the same way within the oral environment (26). Mechanisms could possibly include the disruption of plaque biofilm formation through competition for binding sites on host tissues and other bacteria, and competition for nutrients.

Several mechanisms have been proposed to explain how probiotics work (Figure 2). For example these bacteria secrete various antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins. The production of antimicrobial compounds inhibits some oral bacteria growth and the amount of probiotic bacteria to compete with the pathogenic agents permits a higher adhesion of probiotics on the sites of mucosa than for pathogenic agents. Probiotics can also modify the surrounding environment by modulating the pH and/or the oxidation-reduction potential, which may compromise the ability of pathogens to become established. Finally, probiotics may provide beneficial effects by stimulating nonspecific immunity and modulating the humoral and cellular immune response (20) (27).

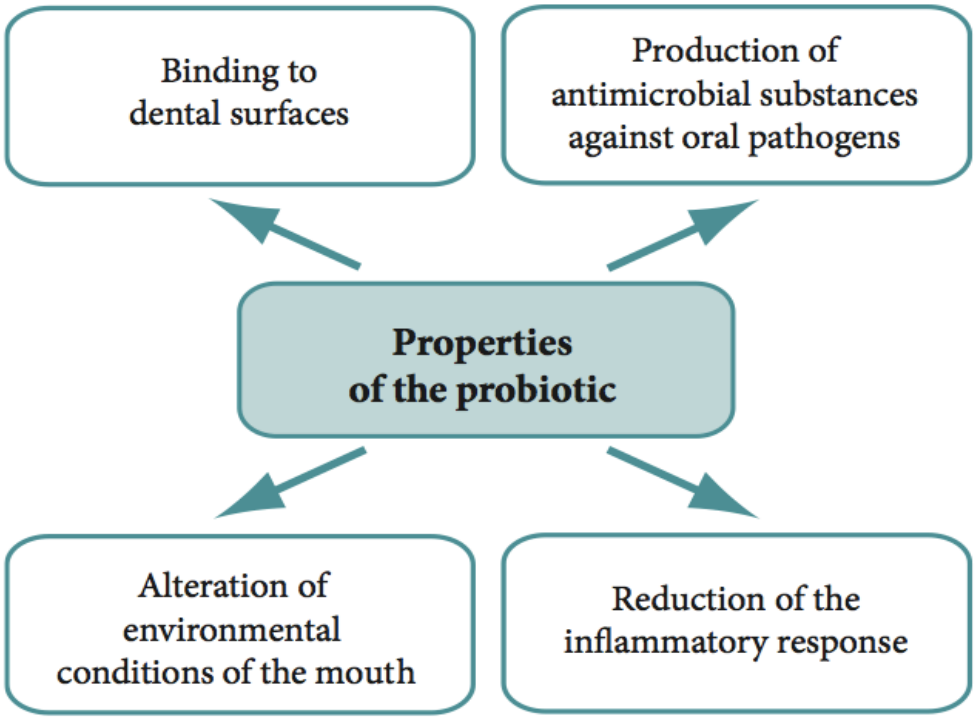

Figure 2. Ideal properties of a probiotic intended for use in disorders of the mouth **(20)**.

Probiotics can, not only suppress the emergence of endogenous pathogens or prevent the infection with exogenous pathogens they may also protect the host through the promotion of a beneficial host response (21). The production of hydrogen peroxide by members of the *Sanguis* group of *Streptococci* induces the reduction of organisms associated with periodontitis. These properties are consistent with the inverse proportions of oral *Streptococci* relative to Gram-negative anaerobes found in dental plaque. Therefore, the implantation of specific oral *Streptococci* or the

encouragement of their growth in dental plaque can be considered a probiotic approach for promoting the shift from a pathogenic to a less pathogenic biofilm (28).

Moreover, there are studies (29) (30) (31) (32) that examined the potential beneficial effect of some oral bacteria selected for their ability to inhibit the growth of pathogens, to down regulate fimbrial expression or biosurfactant production, for the nonappearance of co-aggregation or because of their high prevalence in periodontal health. *Streptococcus sanguinis*, *Streptococcus salivarius* and *Streptococcus mitis* appeared to be the bacterial species most effective in inhibiting periodonto-pathogen colonization *in vitro*. The inhibition is partially caused by environmental conditioning, bacterial interactions, and interaction with epithelial cells (21). *S. salivarius*, is known to produce bacteriocins, which could contribute towards decreasing the number of pathogenic bacteria (33) (34). The existence of probiotics in the indigenous oral microflora of humans permits exploration because these bacteria offer the advantage of being perfectly adapted to the human oral ecosystem (20).

So, the use of probiotics is an interesting emerging field in general and specifically in oral healthcare. Although various "statistically significant" improvements have been reported, but the knowledge of pathogen-host interactions and the role of benificial bacteria in preventing the emergence of pathogenic species and oral health remains obscure. There are great needs to elucidate the role of the oral beneficial microbiota and how the growth can be controlled, which requires making studies on the usefulness of probiotics to maintain or improve oral health (21).

1.2.2. Biofilm and bacterial interactions

Biofilm present on the tooth surface may be among the most complex biofilmthat exist in nature. This is due, in part, to the non-shedding surface of the tooth, which allows for the development of persistent colonization and very complex ecosystems. There is a dynamic co-existence between commensal and pathogenic bacteria and beneficial bacteria, which are protected from the natural physical and chemical antibacterial host defences in these communities (5) (35). There are several areas in oral environment that can be covered by a complex microbial community embedded in an extracellular matrix composed of polysaccharides, nucleic acids, proteins, and water, generally known as oral biofilm (36). By definition biofilm is considered a complex assemblage of microbial cells that are irreversibly attached to a surface and enclosed within a self produced protective polymeric matrix. Biofilms can form on diverse surfaces and can involve single or multiple microbial species. Usually a biofilm is highly resistant to conventional

antibiotics comparing with planktonic from of growth (37) (16).. The oral communities and consequently these biofilms can tolerate antimicrobial concentrations of 10-1000 times that the ones needed to kill planktonic counterparts and displays an inherent resistance to phagocytosis (38) (39).

The precise mechanism for antibiotic resistance remains unclear, however it is likely to be a manifestation of multiple factors. Firstly, the exopolymeric matrix secreted by biofilm bacteria plays a vital role in restricting the penetration of antimicrobials and antibodies (40) (41). Furthermore, negatively charged molecules within the matrix are capable of binding to antimicrobial agents (42). Secondly, bacteria deeply embedded within a biofilm exhibit a reduced growth and metabolic rate and thus are less permeable to antibiotics. Thirdly, inactivation of antibiotics can occur either on the biofilm surface or within the matrix itself (43) with a drug-inactivating of enzymes, such as β-lactamase that causes the degradation of β-lactam antibiotics, and its retention in the dental biofilm amplifies its barrier function (44). Moreover, there may be subpopulation of drug resistant, phenotypically and genetically different bacteria within the biofilm, as the close-knit community provides the ideal niche for the exchange of extra-chromosomal DNA (42).

Biofilm formation is the result of a succession of events, which are very well organized and complex, including the adhesion and the multiplication of the bacteria (45) (46) (47). Plaque or biofilm formation in oral cavity is described as one of highest ordered sequence of events in biofilm formation.

The process starts with acquired pellicle formation and reversible adhesion involving weak long-range physicochemical interactions between the cell and surfaces. With the formation of the pellicle, eventually, the interaction leads to stronger adhesion receptors, that mediate attachment and a succession of co-adhesions which can occur resulting in attachment of secondary colonizers to the bacteria that already are attached. Consequentially, there is bacterial proliferation and biofilm formation and eventually some detachment can happen (35) (16).

The development of a microbial community is initiated by a pioneer microbial population present on oral habitat. These microbial populations have the ability to modify the habitat and thus, new populations may develop (15). These Early colonizers, such as many oral *Streptococci* and *Actinomyces*, have the capability to bind to proteins named adhesins such as alpha-amylase, proline rich proteins, and proline rich glycoproteins that bind to receptors present on glycoproteins (e.g. mucin) in the conditioning film at oral surfaces. *Steptococcus* species, such as *S. sanguinis, S. oralis, S. gordonii, S. mitis* and *S. sobrinus* represent 60 to 80% of all primary colonizers, which

also include 5-30% species of *Actinomyces naeslundii, Fusobacterium nucleatum, Capnocytophaga ochraceae*. Different adhesins are present in the adherence of *Streptococcus* species and acquired pellicle. *S. sanguinis* and *S. oralis* possess adhesins similar to lectine cellular membranes, which are called lectins. Additionally, *S. gordonii* presents more than one adhesin that binds at least to three receptors, namely proline-rich proteins, salivary agglutinins and saliva amylase (48) (49).

Several diagrams show the different "congregate" pairings between the bacteria found in the construction of dental plaque. In Figure 3 it is possible see a simplified diagram about this bacteria aggregation (46) (50).

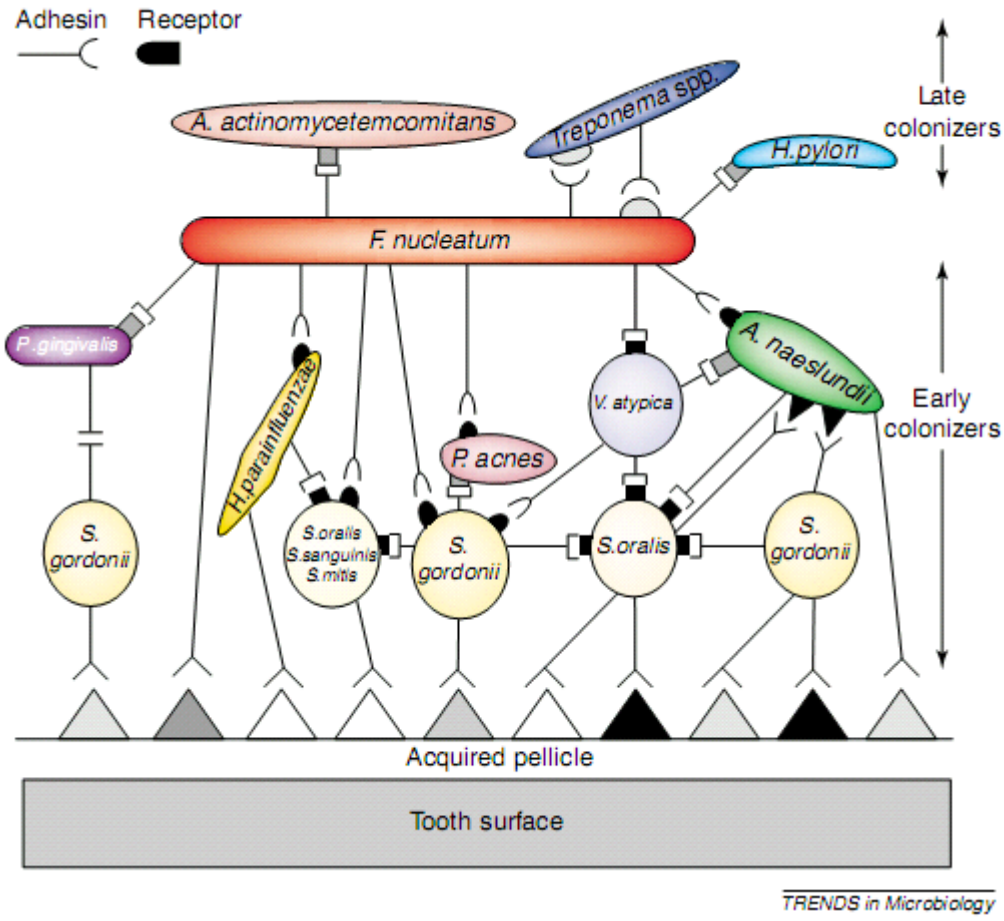

Figure 3. Representation of bonding of oral bacteria on the tooth surface. The complementary sets of adhesin-receptor symbols (an example is shown at the top). Identical symbols are not intended to indicate identical molecules, but they are related functionally **(46)**.

The partnerships between dental plaque bacteria are highly specific and primary colonizers can interact and connect with each other but not usually with secondary colonizers. However, the major periodontal pathogen *P. gingivalis*, a secondary colonizer, can connect with primary

colonizers. One of these early colonizers is *F. nucleatum* that is proposed to be a bridge organism because it can bond with both primary and secondary colonizers (51). In the absence of *F. nucleatum* many other secondary colonizers cannot become part of dental plaque community (52). Additionally, anaerobic secondary colonizers cannot survive in planktonic plaque unless congregated to *F. nucleatum*. Thus, the multiplicity of its congregation interactions and it role as a bridging organism could make *F. nucleatum* an essential organism in the development of dental plaque (46).

Successful colonization of new environments requires several important factors including nutrient supply, an environment conducive to proliferation and an environment with limited potential hazards. Biofilms develop in a vast array of differing environments and thus the structural composition of the biofilm and the extracellular polymeric substances will vary accordingly. The ability to incorporate hydrogen bonding makes the exopolymeric matrix a high hydrated structure. In addition to polysaccharides and water, a wide variety of proteins, glycoproteins, glycolipids and extra-cellular DNA are also present (53).

Bacteria establish interactions between individual cells within one population or between different bacterial populations forming a diversified and complex community, and between them positive and negative interactions can occur. The balance of these interactions is responsible for maintaining the ecological homeostasis within the community. The positive interactions include the relationship between two species where both will benefit from the association (mutualism), when only one of the species benefits whereas the other one obtains nothing from the association (commensalism), and when the interaction between the two microbial species have a greater effect than the sum of the effect of both species taken individually (synergism - mutual adhesion, nutrient cross feeding, complementation in macromolecule hydrolysis, defences against host antibacterial factors). On the other hand the negative interactions include competition, characterized by two populations competing for multiplication and survival and they try to occupy a particular site or obtaining specific nutrients and antagonism, as example when a bacterial population secretes products (hydrogen peroxide, bacteriocin, organic acid) that inhibit other populations or negatively alter environmental conditions (pH, oxidation-reduction potential) (28).

According to what was detailed previously, biofilm microorganisms are held together and protected by a complex matrix of excreted polymeric compounds, the exopolymeric matrix. This matrix functions mainly to protect the microorganisms within, as well as to facilitate intercellular communication (37). This means, microorganisms within a biofilm community actively

communicate through a cell-to-cell signalling system. Therefore, a chemical communication process among them, known as *quorum sensing*, represents an important bacterial function. *Quorum sensing* is defined as gene regulation in response to cell density, and it influences biofilm formation, acid tolerance, and virulence. *Quorum sensing* can occur within a single species as well as between diverse species, and is known to regulate different processes, essentially serving as a simple network communication.

Other way of interaction and communication is by metabolic communications. Saliva, gingival crevicular fluid, food containing sugars, food debris, and metabolic products of other bacteria are the sources of nutrients for oral bacteria and the excretion of metabolites by microorganisms can be used as a nutrient by different species, or the breakdown of a substrate by the extracellular enzymatic activity of one organism, producing available substrates for different organisms, represent metabolic communications among oral bacteria (54). Regulation by inhibitory metabolites is also a kind of interaction. Some bacteria are able to produce bacteriocins that are proteinaceous bactericidal substances that inhibit the growth of closely related bacterial species or strains. The competition through bacteriocin production has been documented for many oral bacteria and this event may regulate the way bacteria interact between them (44).

The oral cavity is exposed to an aerobic environment, so it is likely that oral anaerobic bacteria encounter residual amounts of oxygen in the early stages of biofilm development and periodontal pocket formation. The survival of anaerobic bacteria depends on the specific tolerance of each species to oxygen (through enzymes such as superoxide dismutase, oxidase/peroxidase, and catalase) and the bacterial interactions within the biofilm community (44). Furthermore, the metabolism of aerobic and oxygen-tolerant, species may reduce the concentration of oxygen to levels that can be detoxified by the need of anaerobic bacteria (16).

Furthermore, the detachment of cells from biofilms is essential to allow colonization of new habitats by these bacteria. However, it is probably the least well-understood biofilm phenomenon (55). Studies say that detachment of biofilm cells can be caused by either external or internal biofilm factors. External forces include physical shearing or erosion, sloughing and increased flow velocity for biofilms at liquid interface. Internal biofilm factors are thought to result from reduced nutritional levels or oxygen depletion. These occur by processes such as quorum sensing, endogenous enzymatic degradation, the release of exopolymeric matrix or binding proteins (37). Dispersal strategies include the shedding of individual daughter cells from a micro-colony, the

release of aggregates of biofilm cells or surface dispersal in which cells move across a surface via gliding or twitching motility (49) (56).

There are several reasons why biofilms are a preferred mode of existence for microorganisms. First of all, the bacteria form biofilms as a means of defence, in response to stressful environments such as high shear forces, host defences and deficit nutrients. Another reason for biofilm formation is because this way bacteria can live in very resistant community and they are able to remain in a favourable niche.

Despite the preference of microorganism to form this type of structures, there are many factors affecting biofilms formation. During all the phases of biofilm formation there are always physical and chemical factors interfering in this process. Regarding the attachment of biofilms to a surface is evident the influence of both physical and chemical factors. Physical properties, such as the topography, roughness of the surface, can increase surface area and hence increase colonization and chemical conditions can also reduce the accumulation of bacterial cells in biofilm. Roughness also provides protection from shear forces but increases the difficulty of cleaning (5) (57). It is the case of dental plaque formation, for example, starts in cracks, grooves and irregularities of the tooth surface or tooth implants where the initial colonizing bacteria are protected. Moreover, supragingival plaque formation, after initial colonization has occurred, was shown to occur more rapidly on a roughened surface (5). In metals such as titanium implants, biofilm formation and consequent plaque accumulation occurs especially around the abutment. This may eventually lead to peri-implantitis, an inflammatory reaction with subsequent loss of osseointegration at the dental implant interface (58) (59), causing a loosening of the fixture and, ultimately, the implant would have to be removed (4) (60). Henceforth, these are two important conditions that seem interesting to study. The chemical composition of a surface also has impact on bacterial colonization since it may contain beneficial or detrimental components. For example the influence of the chemical composition of a surface in biofilm formation is related with the dental pellicle on the teeth that may coat the surface and influence colonization. The role of conditioning films on microbial attachment is unclear, but it has been proposed that the strength of the biofilm depends on the cohesiveness of the conditioning film rather than direct bacterial contact with the bare surface (57). The liquid medium surrounding the surface, for example, saliva surrounding the teeth, also influences bacterial attachment and biofilm morphology (5). The existence of various different micro-areas, e.g. tongue, teeth, restorative materials, and gums,

micro-gaps and retentive areas at dental implant interfaces are the most susceptible areas for oral biofilm formation (61).

Moreover, the use of dental implants has been increasing exponentially over the last years, reinforcing the need of their study regarding microbial colonization. One of the most used materials in dental implants is the Titanium (commercially pure titanium), the interest in this material have been increasing over the years. Titanium has excellent proprieties such as good corrosion resistance, biocompatibility, low density, low thermal conductibility, good resistance, low weight and low cost. This material can be submitted to diverse treatments and casting techniques in other to have a better performance. So, the control of the surface is important because this fact affect, in large scale, the adhesion and biofilms formation particularly in plaque-related biofilms (62).

Other important effect is the presence of fluoride on oral environments. Current evidence indicate that fluoride has a multitude of direct and indirect effects on bacterial cells. These include inhibitory effects of fluoride on glycolysis and transport of carbohydrates, enzyme activities, macromolecular synthesis and polysaccharide formation and degradation. Fluoride is well documented as an anticariogenic agent, that involves a variety of mechanisms including demineralization, the enhancement of remineralisation, the interference of pellicle and plaque formation and the inhibition of microbial growth and metabolism (63). So, fluoride has been used to help the control of incidence of caries and to decrease the dentin sensibility and oral plaque formation. The results of many studies confirm that fluoride from the substratum affected fluoride-sensitive biofilms and reduce the risk of plaque formation responsible for many oral diseases (64) (65) (66).

1.2.3. Relation between oral health and other diseases

It has been suggested that there is association between the oral microbiota and other diseases, such systemic diseases, cardiovascular disease (including coronary diseases, myocardial infarction, bacterial endocarditis), complications during pregnancy, chronic diseases (e.g arteriosclerosis) and aspiration pneumonia diseases (67) (68) (23) (10) (11) (69) (70) (71).

The relationship between oral health, specifically periodontal disease, and cardiovascular and respiratory diseases has been subject of ongoing research and supported by many studies that have reported the association between these diseases and periodontal infections. Studies show that infections can be caused by periodontal pathogens like *Aggregatibacter*

actinomycetemcomitans and *P. gingivalis* and these may be associated with future stroke, increased risk of myocardial infarction, and acute coronary syndrome (72).

Chronic infections start with inflammation, so periodontitis and gingivitis might influence systemic or/and vascular inflammation processes. The source of bacterial pathogens responsible for the most prevalent chronic infections affecting humans, dental caries and periodontal diseases, derives from the biofilms present in tooth surfaces. These biofilms are one of the most complex existing in nature, due in part to the non-shedding nature of tooth surfaces that allow the development of a persistent bacterial colonization and to the rather complex ecosystems that exist in the oral cavity (48) (3). Bacteria themselves, once in the blood stream may cause distant site infections and the products from bacteria can stimulate systemic inflammation that would eventually act directly and/or indirectly on the vascular walls inducing a state of endothelial dysfunction

So, bacteria that reside in the subgingival biofilm may disseminate systemically and influence directly or indirectly the site of inflammation causing these type of systemic diseases.

In other to avoid greatest health problems, treatment of oral diseases should start as soon as possible. Treatment can affect bacteria directly by physical removal and/or with chemotherapeutic agents (Figure 4) (3).

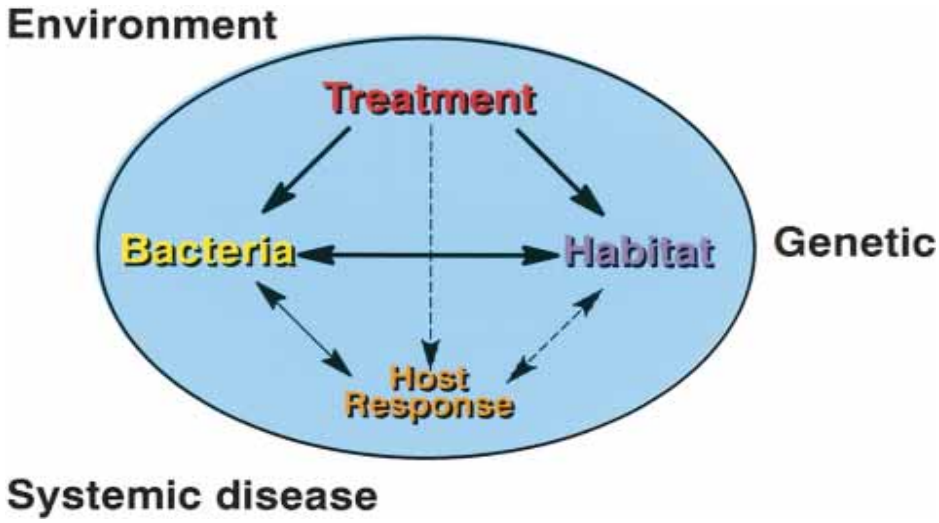

Figure 4. Diagrammatic representation of the effect of therapy on colonizing bacteria, the host and the habitat. **(5)**.

Dental biofilms can be altered by various therapies providing a beneficial outcome to the patient and treatment can affect the composition of the bacterial plaque directly, can affect the

host response or alter the habitat for example, by eliminating or by meticulously removing supra-gingival plaque. Alterations of any of these factors can impact on the remaining factors.

Therefore, improvement of oral hygiene has been shown to reduce the occurrence of these diseases. The consequences of poor oral health linked with advanced age, common co-morbidities such as diabetes, concurrent medications and a state of immune dysfunction that may increase the risk for systemic consequences of periodontitis and other oral and dental pathologic conditions. Thus, oral hygiene assumes an important role in the care of high-risk subjects.

Besides, colonization by pathogenic periodontal bacteria is a risk factor for an implantation of a periodontal implant and the accumulation of biofilms can promote periodontal inflammation of the mucosal soft tissues surrounding the implant, while peri-implantitis also affects the supporting bone in the subgingival area, resulting in a rapid bone loss. Peri-implant diseases (peri-implantitis and peri-implant mucositis) are major clinical problems that appear in patients possessing osseointegrated dental implants that can, ultimately, result in the loosening of the implant (73).

2. Chapter II

The use of dental implants has been increasing exponentially over the last few decades, making it essential to understand how bacteria grow and react in these materials. Thus, the goal of this chapter is the evaluation of the effect of different types of Titanium surfaces in microbial colonization, namely biofilm formation. Moreover, it also aims to study the influence of the presence of fluoride and probiotic bacteria on pathogens' biofilm formation.

2.1. Introduction

Osseointegrated titanium implants have become an important alternative to conventional prostheses, increasing significantly the quality of life of patients. On the other hand, with the increasing demand for dental implants, its failure is also being reported more frequently (74) (75) (76) (77) (78). Nowadays, oral implants are used not only to replace missing teeth, but also to provide anchorage during orthodontic treatments, to rebuild the craniofacial skeleton or simply for aesthetic circumstances (79). It is estimated that more than 2 million dental implants are placed annually (73). Thus in the near future an increase of this number is expected not only due to an increasingly aged population, but also because implant therapies have become highly successful (80), with implant survival rates above 89 % after 10/15 years (81).

However, it is still necessary to study and understand the causes of dental implant failures. These are defined as implants that exhibit clinical mobility, pain on function, bone loss more than half of the total length of the implant, or uncontrolled exudates (82). The timing at which implant failures occur represents different physiological processes. An early implant failure indicates an initial lack of osseointegration due to an inability to establish an intimate bone-to-implant contact. Various factors may contribute to early implant failures such as premature loading, surgical trauma, or impaired healing response (83). Late failure, on the other hand, occurs after initial integration, physiological remodeling and loading. Causes of late failures include overloading and bacterial infection (e.g., peri-implantitis) with most failures occurring after the first year of loading. In fact, biofilms have been associated with almost 65% of infectious diseases such as periodontal and peri-implant diseases leading to implant failure (84) (85).

The role of bacterial biofilm in peri-implant diseases has been recognized, so, the knowledge on the microbiology around dental implants is essential of the essence for adequate diagnosis and treatment of these diseases. Thus, this chapter focuses on understanding the development of oral biofilms in titanium samples that are used in dental implants and some interactions between periodontal bacteria.

2.1.1. Formation of biofilm on dental implants

The oral cavity represents a perfect fluid system in which the microbiota, present in saliva, may colonize on teeth and artificial surfaces following the deposition of a glycoprotein-containing pellicle (86). This pellicle is derived from components in the saliva, as well as bacterial and host

tissue products. It acts as a substrate for bacterial colonization, which occurs as early as 30 minutes after implant exposure in the oral cavity (87). The pellicle is formed after the exposure of an implant in the oral cavity through a transmucosal abutment. Then, the selective adsorption of the environmental macromolecules such as a-amylase and serum albumin occurs (88).

In comparison to natural teeth, the acquired pellicle on dental implants has a lower albumin adsorption capability, which according to some authors (85), contributes to the lower plaque formation around implants.

Some studies have shown that an increase in titanium surface area structure and the surface free energy also facilitates the formation of bacterial biofilms (9). Both adsorbed salivary proteins and implant surface structures contribute to the early colonization of oral titanium implants (89) (90) (91) (76). If bacteria can attach themselves directly to an inert titanium surface, this may have consequences leading to infection of the peri-implant tissues (92).

It is well known that the formation of bacterial biofilm is an important factor in the infection of medical devices (93). It appears from other studies that at least the transmission of *Porphyromonas gingivalis* and *Prevotella intermedia* from the periodontal pocket to the peri-implant region is possible (94). Data suggests that shortly after the installation of titanium implants a sub-gingival microbiota dominated by *Peptostreptococcus micros*, *Fusobacterium nucleatum*, and *P. intermedia* is established (95). In addition, recent data suggest that colonization on different sub-implant surfaces occurs quickly (96) (97) (87) (32).

The infectious aetiology of peri-implantitis is well established (i.e. Mombelli et al. 1988; Roos-Jansa ker et al. 2003) and studies have shown that high levels of periodontal pathogens such as Aggregatibacter actinomycetemcomitans (former Actinobacillus actinomycetemcomitans), *Porphyromonas gingivalis*, *P. intermedia*, *Tannerella forsythia* and *Treponema denticola*, have been associated with peri-implantitis (75). Peri-implant infections may also include *F. nucleatum* and *actinomyces* species (98). In addition, *Staphylococcus aureus* and *enterococci* spp. have been related to peri-implant infections (99). Whether the surface characteristics of titanium implants influence the microbiota is poorly understood.

It is possible that unknown bacteria are involved in the emergence of the lesions and the pockets around the remaining teeth may act as a bacterial reservoir. The composition of the peri-implant microbiota is likely to be similar to that around teeth. However, few studies have evaluated the differences in bacterial composition between dental implants and remaining teeth in the same subjects (74).

Recent studies indicate that soon after dental implantation and/or restoration, biofilm formation and consequent plaque accumulation occurs on titanium implants, especially around the abutment. This may eventually lead to peri-implantitis, an inflammatory reaction with subsequent loss of osseointegration at the dental implant interface (59) (4), causing a loosening of the fixture and, ultimately, the implant would have to be removed (100).

In conclusion, biofilm formation on medical implants presents three major problems, which in the end can lead to implant failure.

2.1.2. Titanium in dental implants

Commercially pure titanium (Ti) and titanium alloys are the first choice for oral prosthesis, mainly due to their biocompability and excellent mechanic characteristics (101).

A range of titanium surfaces with different composition and roughness has been developed for dental implants. Moreover, the topography of titanium dental surfaces is of major importance for microbial colonization (90) (91) (76). However, much controversy still exists as to the optimal features for implant surfaces regarding both osseointegration and antimicrobial kinetics. In addition, higher surface roughness profiles may as well lead to an increase in ionic leakage to adjacent tissues, thus presenting major risks (102).

Increased surface roughness has been associated with increased osseointegration of the dental implant (103). Conversely, a higher surface roughness increases biofilm formation (91), and thus contributes to spontaneous progression of peri-implantitis lesions (104).

Additionally, titanium also denotes a strong osseointegration tendency by the development of close bone-to-implant apposition after short periods of implantation, an important feature for permanent bone-interfacing implants. However, an ideal dental implant material should not only integrate with the host tissue, but also exhibit anti-bacterial properties (105).

2.1.3. Influence of Fluoride in biofilm formation

The effect of fluoride on oral bacteria has been studied extensively over the last 20 years and the current evidence indicates that fluoride has a multitude of direct and indirect effects on bacterial cells. These include inhibitory effects of fluoride on glycolysis and transport of carbohydrates, enzyme activities, macromolecular synthesis, and polysaccharide formation and degradation. Studies, both *in vivo* and *in vitro*, have been made in an attempt to elucidate the

potential for these actions of fluoride to contribute to the control of plaque and caries. Various models have shown that fluoride may influence plaque accumulation, acid production and enamel demineralization (64).

The antimicrobial activity of fluoride is well documented by a considerably amount of literature (106) (107) (108). The mechanisms by which fluoride may interfere with bacterial metabolism and dental plaque acidogenicity. Furthermore, intracellular or plaque associated enzymes such as acid phosphatase, pyrophosphatase, peroxidase and catalase may be affected by fluoride ions. Although even low fluoride levels may reduce bacterial growth and formation of dental plaque but the affection of plaque metabolism by fluorides is still unclear (109) (107).

2.1.4. Treatment of dental implant-associated infections

Treatment of infections associated with dental implant and biofilms consists in mechanical debridement of the implant surface or chemical treatment including local and systemic antibiotics.

The selection of treatment depends on the established diagnosis of peri-implant mucositis or peri-implantitis. Treatment success is assessed using outcome measures, such as reduction of inflammation, probing depth, and pathogenic bacteria (110). Nonetheless, the presence of specific bacteria had little or no value in predicting treatment failure (111).

In a recent literature review, non-surgical mechanical therapy was effective in treating peri-implant mucositis with improved results observed in conjunction with an antimicrobial mouth rinse (84). A reduction in the proportion of pathogenic species after mechanical therapy has been reported (100).

However, nonsurgical treatment of sites with peri-implantitis was not found to be effective at reducing inflammation, pathogenic microorganisms, and bleeding on probing. The addition of antimicrobial mouth rinse in this nonsurgical treatment of peri-implantitis only provided minimal beneficial effects (82) (84). On the other hand, the use of local drug delivery such as minocycline and tetracycline to treat peri-implantitis generated reduced levels of *T. forsythia*, *P. gingivalis*, and *T. denticola*, with the most effect on A. actinomycetemcomitans (85).

In the past decades, laser therapy such as diode, CO_2, and Er:YAG laser as gained popularity based on the rationale of surface decontamination, hemostatic properties, calculus removal, and bactericidal effects (112) (113). However, only minor clinical and microbiological improvement has been reported (84).

These are some the conventional treatments and therapies. There are numerous studies purporting others protocols to improve oral health and decrease the mentioned oral diseases (85).

2.1.5. Probiotics as improvement for oral health

Probiotics have been found to be beneficial to the host by improving the endogenous flora. Traditionally, probiotics have been associated with gastrointestinal tract, however recently several lines of research have suggested use of probiotics for oral health (20).

There are a numerous reasons why probiotic research has become a famous topic in medicine. Despite over 50 years of antibiotics, infectious diseases remain a major health problem, creating multi-drug-resistant bacteria, while pathogenic microorganisms are being linked with induction or worsening of many chronic diseases. Moreover the alarming spread of infectious diseases, leads scientists and industries to look for new approaches to health restoration and retention. Science itself is playing a major role, with an ever-growing number of studies providing concrete evidences that probiotics can alleviate some disease processes (114) (29).

In the field of periodontal healthcare, probiotics might provide opportunities related with the current view on the aetiology of plaque-related periodontal inflammation. This aetiological view considers three factors that determine whether disease will develop in a subject: a susceptible host, the presence of pathogenic species and the reduction or absence of some bacteria called ‘‘beneficial bacteria’’.

It is difficult to influence the host response because traditional periodontal therapies are focused on the reduction of the bacterial threat using mechanical technics or chemical treatments (115). These applied treatments strategies are based on a mechanical subgingival debridement or use of local or systemic antibiotics, in combination with improved oral hygiene (116). These changes the subgingival microbiota to a less pathogenic composition, which is characterized by high proportions of Gram-positive aerobic species and low proportions or absence of periodontopathogens (117). Unfortunately, it is currently unclear the necessary decrease of the proportion of pathogens or the necessary increase on Gram-positive aerobic species needed to increase to consider a subgingival biofilm as not pathogenic (29). The real question is related with the duration of the treatment in other to consider that the amount of pathogenic bacteria is safe and the equilibrium between pathogenic and probiotics is already enough.

In the oral cavity, probiotics can build a biofilm, acting as a protective coating for oral tissues against oral diseases. This biofilm keeps bacterial pathogens out of oral tissues by occupying a site

that pathogens would invade in the absence of the biofilm and competing with cariogenic bacteria and periodontal pathogen growth (118).

So, probiotic bacteria can provide health benefits to the host by: providing nutrients and cofactors to the host, competing directly with pathogens, interacting with the pathogen virulence factor and stimulating the host immune response (119).

Usually streptococci, lactobacilli, or bifidobacteria are included in probiotics which not only suppress the emergence of endogenous pathogens or prevent superinfection with exogenous pathogens.

Recently, it has been reported that probiotics could protect the periodontium through the promotion of a beneficial host response. In other words, when *Streptococcus salivarius, Streptococcus sanguinis,* and *Streptococcus mitis* were used, this could reduce the interleukin-8 epithelial responses to *Aggregatibacter actinomycetemcomitans* (formally *Actinobacillus actinomycetemcomitans*) (120) (21).

However the mechanism behind the successful inhibition of periodontopathogen recolonization remains hypothetical. Several possibilities such as, the occupation or a physic-chemical alteration of the subgingival niche, competition for essential nutrients, inhibition of the viability or growth of pathogens, and modification of the production or degradation of virulence factors of pathogens or immune responses, are being considered as the main underlying mechanisms (120)

2.2. Materials and Methods

2.2.1. Sample preparation

Commercially pure titanium (Ti) (grade 2) (Goodfellow Cambridge Limited, England) samples were cut from the same original plate in square form (20x20x2 mm) and were gently provided and cleaned by the Research Group on Functionalized Materials and Surfaces Performance of University of Minho. Grade 2 titanium was selected as the most common grade of titanium used in dentistry. Two different surfaces topographies were used: Etched and Anodized. Etched Samples were cleaned during 15 min in warm water (60°C) in an ultrasonic bath. For the anodized samples the cleaning process consisted out of an ultrasonic bath with propanol for 10 min and a 5 min of immersion with distilled water.

All the samples were dried at room temperature and kept in a desiccator. Before each assay samples were sterilized in a steam autoclave at 121 °C for 20 min, at 1 atm.

2.2.2. Bacterial Culture

In other to create a bacterial multispecies environment for biofilm formation three different pathogenic oral bacteria, *Prevotella intermedia* (ATCC 25611), *Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (ATCC 10953), were used. In some assays one probiotic, *Streptococcus salivarius* (ATCC 7073), was also used. These bacteria were maintained on blood agar plates (Blood Agar Base II, Oxoid, Basigstoke, UK) supplemented with 5% of horse blood (Biotrading, Keerbern, Belgium). *Streptococcus salivarius* was incubated at 37°C in an atmosphere with 5% of CO_2. While for *P. intermedia, P. gingivalis*, *F. nucleatum* growth was performed in anaerobic conditions in anaerobic jars containing 80% N_2, 10% CO_2 and 10% H_2 (Anoxomat, the Netherlands) which were incubated at 37°C.

One day before the each experiment, the bacteria were collected from the blood agar plates and were grown overnight in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, USA), in the same conditions.

2.2.3. Culture media and solutions

An artificial saliva solution was used in this work, as culture medium for the biofilm formation (Table 1).

Table 1. Composition of artificial saliva

Compounds	Concentration
BHI broth	37g/L
Lab Lenco beef extract	1g/L
Yeast Extract	2g/L
Peptone	5g/L
Sodium chloride (NaCl)	5g/L
Mucin	2g/L
Urea	40% solute 1,25mL/L
Potassium chloride (KCl)	0.2g/L
Calcium carbonate	0.3g/L
Magnesium chloride	0.2g/L

The culture medium was prepared by dissolving all the components, mentioned in the table 1, in distilled water.

To evaluate the effect of fluoride (F^-) in biofilm formation, in the specific assays, a solution of sodium fluoride (NaF) with a concentration of 0.5g/L was added to the artificial saliva medium, simulating fluoride concentrations in the oral cavity[].

In order to distinguish the different bacteria, several selective media were prepared:

(i) phenylethyl alcohol agar (PAA) enriched with 5% defribinated horse blood - *S. salivarius*;

(ii) Crystal Violet Erythromycin agar plates (CVE) (Difco, Detroit) - *F. nucleatum*;

(iii) blood agar plates (Difco, Detroit) enriched with 5% defribinated horse blood, 10 µL/mL haemin, 1 µL/mL menadione and 30 mg/L gentamycine, L-Cystine 0.4g/L, kanamycin 0.1g/L and Vancomycin 7.5mg/L (BAKV) - *P. intermedia*;

(iv) blood agar plate (Difco, Detroit) enriched with 5% defribinated horse blood, 10 µL/mL haemin, 1 µL/mL menadione and 30 mg/L gentamycine (BAP) - *P. gingivalis*.

All growth culture media were sterilized in a steam autoclave at 121 °C for 20 min, at 1 atm, right after the addition of all the components.

2.2.4. Biofilm formation

Mixed biofilms of the pathogenic bacteria were formed on both types of samples (etched and anodized), in order to investigate the influence of anodization processes on oral bacterial biofilm formation. Furthermore, the influence of the presence of fluoride and the presence of a probiotic bacteria (*S. salivarius*), on the pathogens' biofilm formation, was also considered. tree conditions were tested for the two types of material:

a) **Control biofilm** composed by *P. intermedia*, *P. gingivalis, F. Nucleatum*;
b) **Biofilm with fluoride** composed by these three bacteria and artificial saliva medium supplemented with NaF;
c) **Biofilm with *S. Salivarius*** composed by *S. salivarius* simultaneously with the other three bacteria (*P. intermedia, P. gingivalis, F. nucleatum)*

After cell incubation, as mentioned previously, the cultures were centrifuged (7500 rpm; 10min) and the supernatants were discarded. The pellets were washed in PBS (pH 7.4) and centrifuged again. Bacteria were then re-suspended and diluted in artificial saliva medium to achieve the concentration of $1x10^8$ CFU/mL using a spectrophotometer and a wavelength of 600 nm.

Sterilized Ti samples were placed into wells from a standard 6-well culture plates and 1.5 mL of the each cell suspension was transferred to each well. The 6-well plates were then incubated in an anaerobic jar with 5 % CO_2 at 37 °C for 8 days under static conditions. Fresh media changes of 2mL were performed every 2 days.

For each assay (a to c) the experiment was performed in triplicate.

2.2.5. Biofilm Analysis

Microbial culturing, crystal violet and scanning electron microscopy (SEM) were used to analyze biofilm formation. To quantify the number of viable bacteria present in the biofilms microbial culturing was used. Crystal violet was used to quantify the total biofilm biomass by absorbance reading. Another completely different analysis was made by SEM that permits the observation of the structure of the biofilms.

2.2.5.1. Crystal violet

After biofilm formation all medium was removed from the wells and Ti samples with biofilms were placed in new wells and washed with 0.9 % NaCl to remove non-attached cells. Biofilms were then fixed with methanol (4mL) for 15mins. Methanol was removed and biofilms were air dried for 15mins. Biofilms were then stained with 4 mL of a 1% (m/v) crystal violet solution, for 5min. The crystal violet staining was aspirated and the remaining biofilm was washed two times with distilled water, to remove excess of stain, and dried at room temperature. The remaining staining was solubilised with acetic acid (4 mL, 33 % in water). Finally, the optical density of the solutions present in each well was measured at 570nm using a spectrophotometer.

2.2.5.2. Microbial culturing

Based on the number of detected colonies another group of titanium samples with biofilms was used to determine the number of viable colony-forming units (CFU) (bacteria/mL) of *P. intermedia, P. gingivalis, F. nucleatum* and *S. salivarius*. After biofilm formation all medium in the wells was removed and the Ti samples with biofilm were washed with PBS (pH7.4) to remove non-attached cells. Ti samples with biofilm were placed to new wells and the plates were incubated with 4 mL in each well of 1% protease (Sigma-Aldrich) during 1hour at 37°C.

Following incubation, the cell suspension of each well were removed to a falcon and sonicated for 5 min to disrupt microbial agglomeration. The initial suspension was diluted in physiological water (with 9% of NaCl) and 50 μl was placed on agar plates to count the total viable CFUs.

Initially, the suspensions were placed only on blood agar plates (Boold Agar Base II, Oxoid, Basigstoke, UK) supplemented with horse blood (Biotrading, Keerbern, Belgium) and it was possible to count the different colonies due to the difference in morphology between *P. intermedia, P. gingivalis, F. nucleatum.* When working with *S. salivarius* it was necessary to monitor the growth with microbial culturing on species-specific agar plates (PAA, CVE, BAKV, BAP).

2.2.5.3. Scanning electron microscopy

Scanning electron microscopy (SEM) allows a detailed observation of the structure of the biofilms. However, its resolution power is not always sufficient, especially when is necessary observe extracellular structures that may be involved in adhesion.

Before observation by SEM, biofilms formed on different adhesion surfaces (etched and anodized Ti samples) were dehydrated by immersion in absolute ethanol solutions with increasing concentrations until 100% (50%, 70%, 80%, 90%) remaining around 5min in each solution. Subsequently, the samples with biofilms were transferred to desiccators for complete dying. After this step, the samples were sputter-coated with gold and mounted on aluminium stubs with carbon tape and examined in a scanning electron microscope (Nova 600 NanoLab, FEI Company, USA) between 10KV and 15KV. The observations were documented through the acquisition of photos.

2.3. Results

2.3.1. Analysis of mixed biofilms formed on etched and anodized Ti samples

The formation of mixed biofilms on different surfaces (etched and anodized) was quantified using the crystal violet (CV) staining method, the enumeration of the number of viable colony-forming units (CFU) and by SEM observation.

The staining with CV, for quantification of biofilm biomass, includes both cells and exopolimeric matrix. observed in Figure 5, biofilm formation varied according to the surface topography, the resultant staining of the anodized samples was stronger than for etched samples.

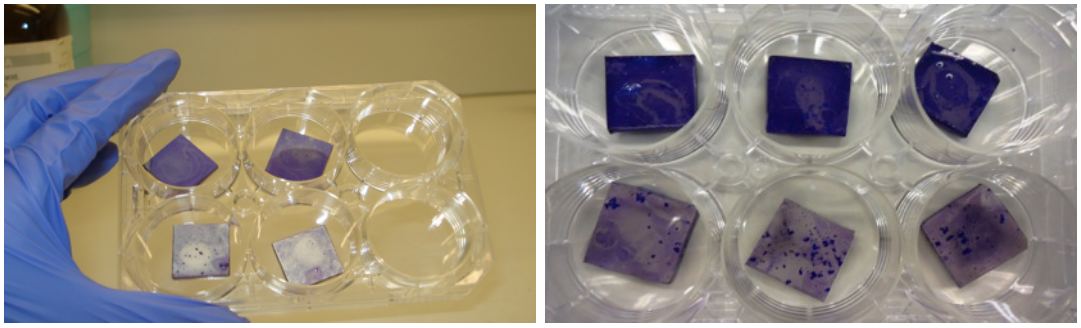

Figure 5. Crystal violet staining of mixed biofilms formed in samples with different topographies: anodized (top) and etched (bottom).

The differences highlighted in Figure 5, were confirmed by the dissolution of CV staining and its spectophotometric quantification (Figure 6). A significant increase of biomass ($p < 0.05$) was found on surfaces with higher porosity (anodized) compared to the etched sample.

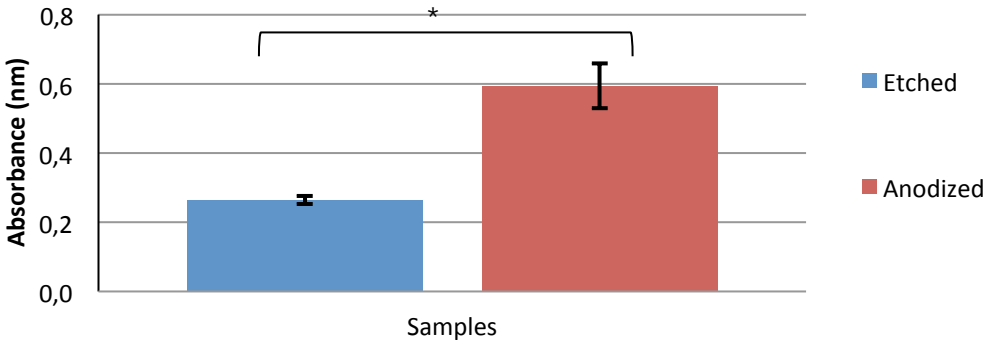

Figure 6. Crystal violet absorbance of mixed biofilm biomass formed on different titanium surfaces (Etched and Anodized). * represents the statistical differences between the two different samples.

Besides the analyses using Cristal violet, the quantity of viable cells of each bacteria in the different samples was determined, using the number of colony forming units (CFU). Initially, the CFU were quantified for PI, PG and FN that were plated on blood agar plates supplemented with horse blood, to confirm their distinction once they presented different morphologies on this same medium (Figure 7).

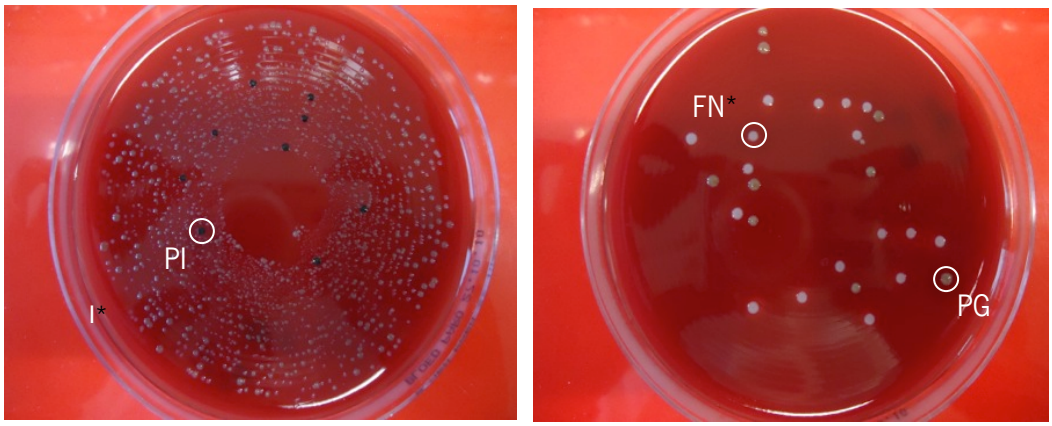

Figure 7. Blood agar plates supplemented with horse blood used to determine CFU of the 3 bacteria (PI - black, PG - green and FN - white).

It was then possible to confirm (Figure 7) that the different bacteria grow with different colony colour. Therefore, in anaerobic conditions and the counting results showed that PI did not grow of the control biofilms (Figure 8), at least not insufficient quantity to be detected for this method. Moreover, PG and FN were both detected on the control biofilms, although in a little less extension for PG comparing to FN, $p<0.05$.

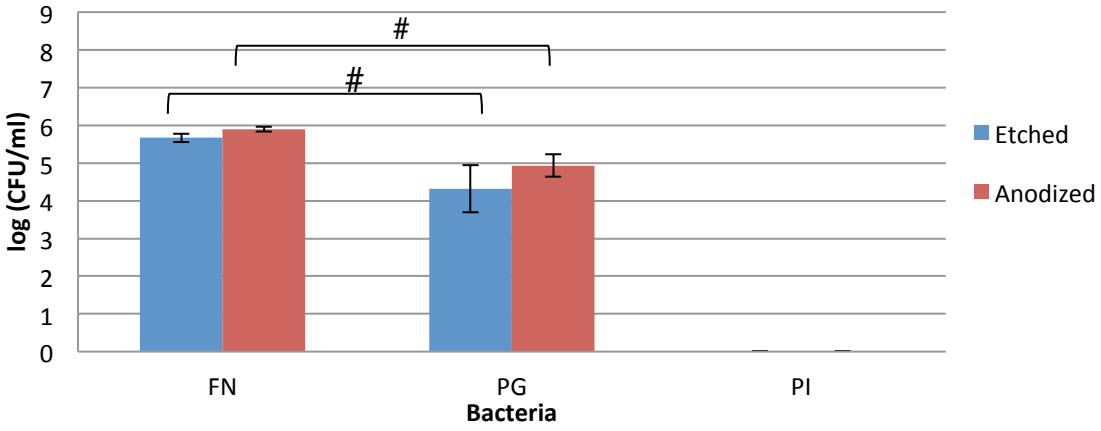

Figure 8. Average values of the colony-forming units of multi-species biofilms of FN, PG and PI that grew during 8 days in anaerobic environment. The error bars represent the standard deviation and # represents the statistical differences between the colony-forming units formed for each bacteria.

In opposition to the results for crystal violet, the CFU determination did not show significant differences ($p>0.05$) between the two types of samples.

SEM analysis was also made in both surfaces to observe the topography of the samples and the biofilms formed on them (Figure 9).

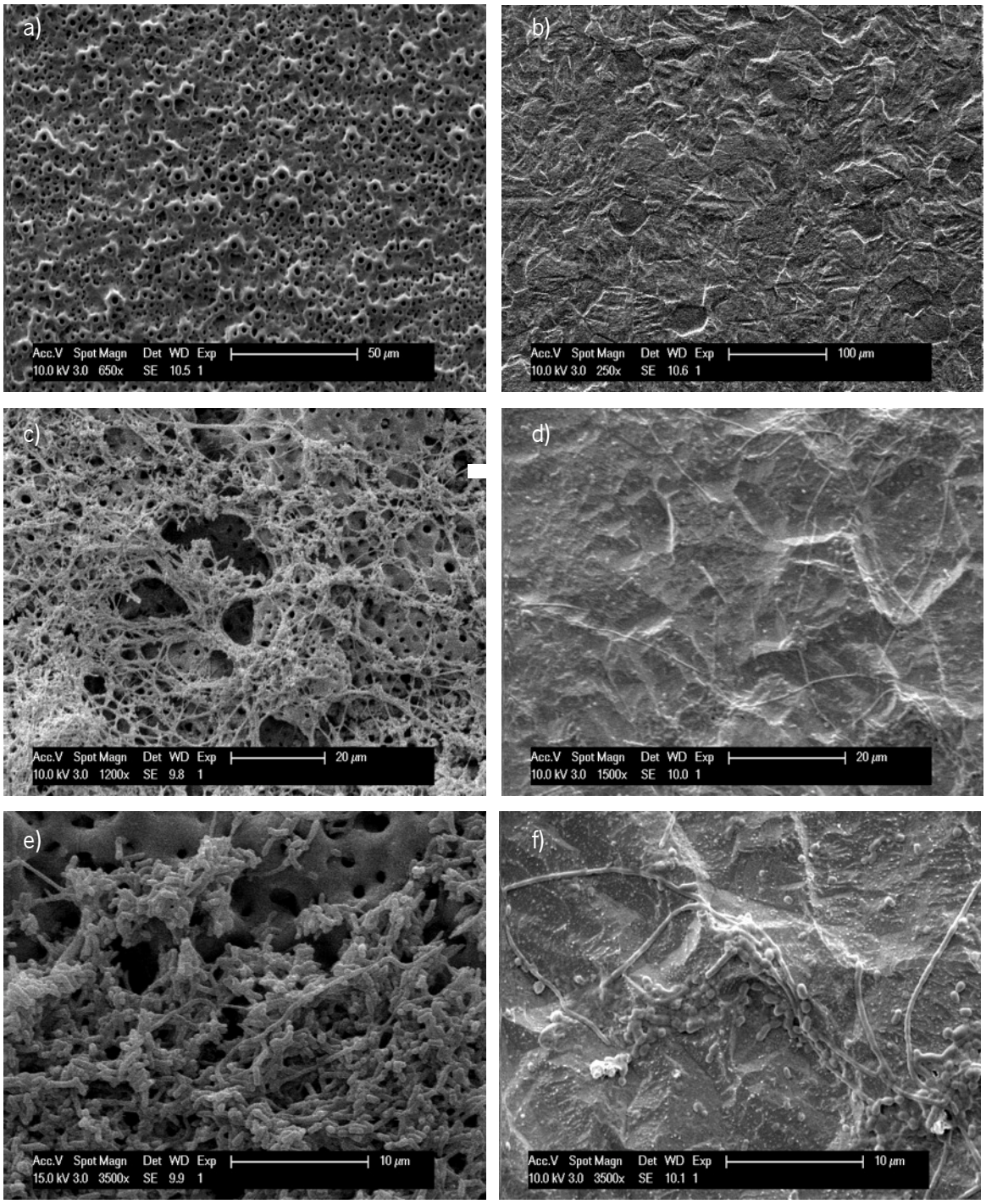

Figure 9. Micrographs of anodized (a, c and e) and etched (b, c and f) Ti samples, obtained by SEM witch c, d, e and f are covered with a mixed biofilm of PI, PG and FN, c and d with a scale of 20 µm and e and f with 10 µm.

The SEM images of samples without biofilms illustrate that the samples have different topographies being the anodized sample more porous than the etched. It is observed that biofilms were formed on both surfaces, but for anodized samples (Figures 9 c and e) the biofilm was formed in larger amount than for etched samples (Figures 9 d and f). These results are in accordance with the results obtained with CV analyses that also indicate a higher formation of biofilm for anodized samples.

However this method is only qualitative and only allows the visualisation of the complexity of biofilms attached to the surface

2.3.2. Evaluation of the presence of fluoride in biofilm formation

In order to assess the influence of fluoride (NaF) on formation of mixed biofilms on anodized and etched Ti samples, the artificial saliva medium was supplemented with fluoride and the cultures with PI, PG and FN were incubated in anaerobic conditions during 8 days. For both samples the presence of fluoride caused a high inhibition (65% and 83%, for anodized and etched samples respectively) of the amount of biomass, in comparison with biofilms formed without fluoride supplementation (Figure 6), indicating that fluoride has a negative influence on growth of oral bacteria and on formation of oral biofilm. Similar results were obtained with the analysis of viable CFU, no CFUs were detected when the medium was supplemented with fluoride (Figure 10).

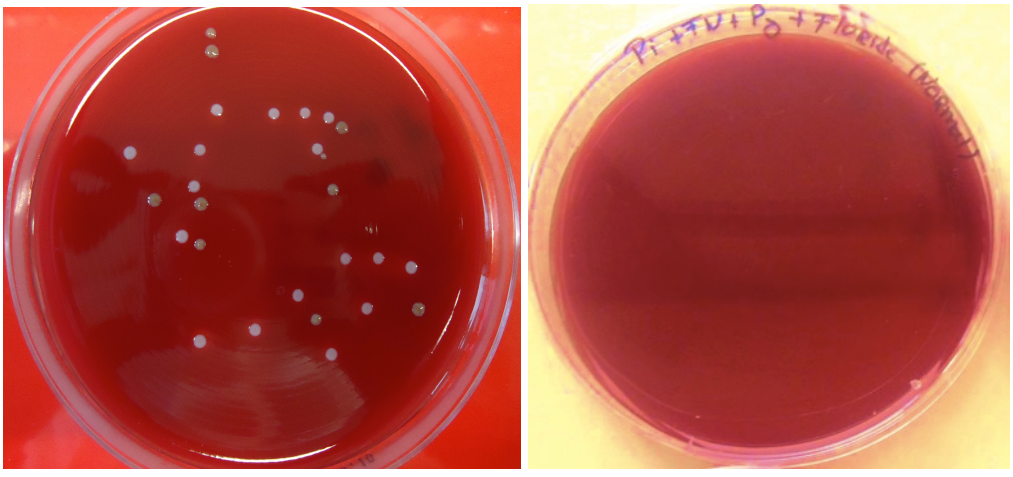

Figure 10. Blood agar plate supplemented with horse blood used to determine CFU of a biofilm formed in TI sample in the absence (left) and presence of fluoride (right).

So, the presence of fluoride seems to avoid biofilm formation by the species assayed and the results revealed that the presence of this substract is a crucial factor for biofilm formation.

2.3.3. Analyses of the effect of the presence a probiotic bacteria on mixed biofilm formation by pathogenic bacteria

It was also intended in this study evaluate the influence of a probiotic bacteria (*S. salivarius* - SS) on biofilm formation of pathogenic bacteria (PI, PG and FN). The resultant biofilms were analysed in terms of biomass (Figure 11) and number of viable cells (Figure 12).

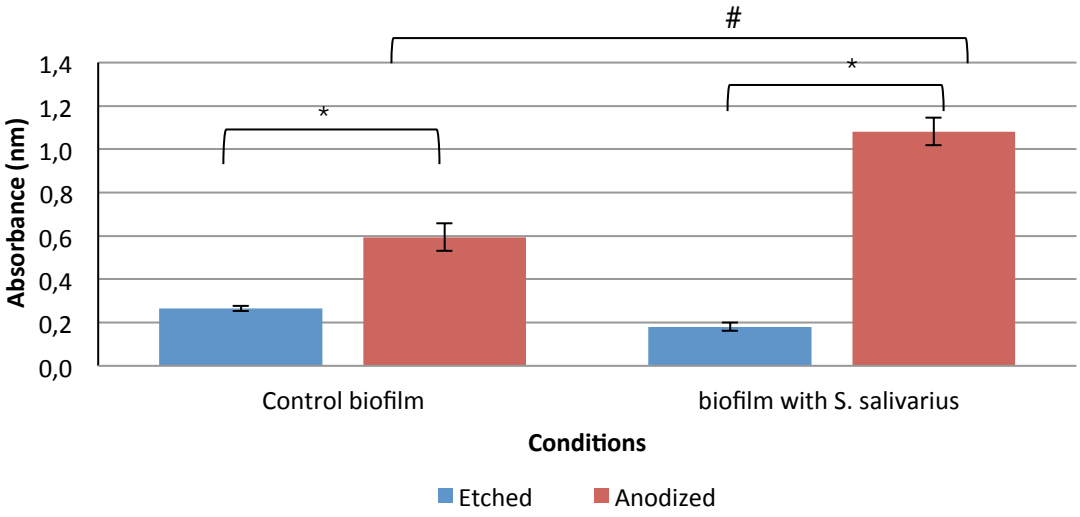

Figure 11. Crystal violet absorbance of mixed biofilm biomass formed on different titanium surfaces (Etched and Anodized) after 8 for control and in the presence of *S. salivarius*. * represents the statistical differences between the two samples, # represents the statistical differences between two conditions in same type of sample.

The use of CV staining allows a quantitative analysis of all the biomass (cells plus extracellular matrix), so, in these assays, with SS, the amount of biomass is greater or almost the same than the ones without SS (Figure 11). Specifically, comparing biofilms formed in the absence or presence of SS there are only significant differences ($p<0.05$) between the anodized samples.

Interestingly, when biofilms were formed in the presence of SS there were differences in the detected viable bacteria (Figures 12 and 8).

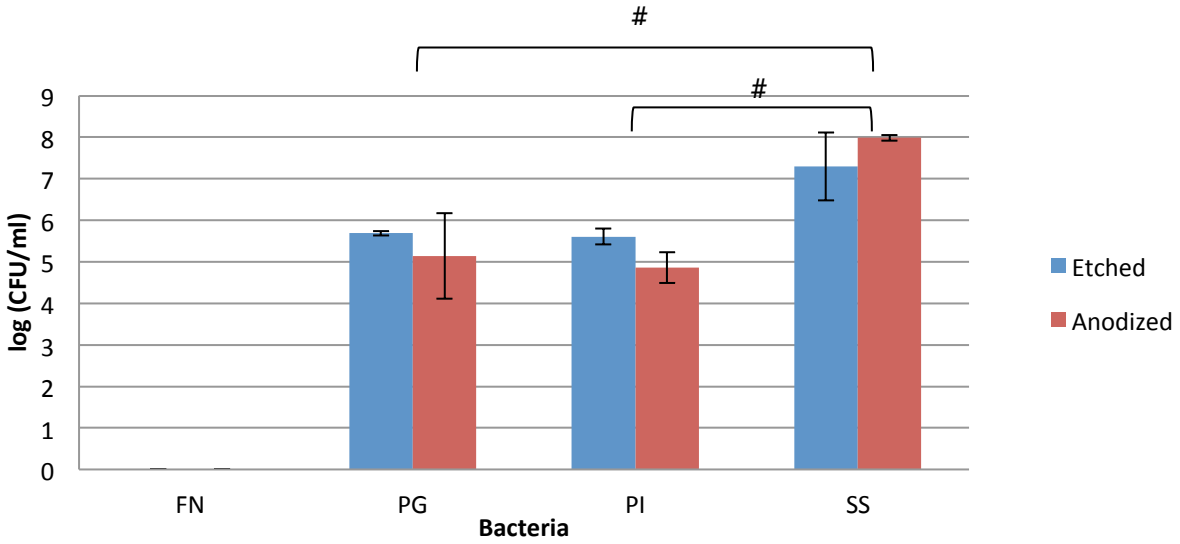

Figure 12. Average values of the colony-forming units of multi-species biofilms of FN, PG, PI and SS, which grew during 8 days in anaerobic environment. The error bars represent the standard deviation and # represents the statistical differences between two conditions in same type of sample.

In the presence of SS, PI was detected but FN was not (Figure 12), in opposition to the control (Figure 8). There was obviously a huge growth of SS, confirming the results of biomass (Figure 11) were the amount of biomass was bigger in the presence of SS than in the control. Interestingly the number of PG was very similar to the amount seen in the control biofilm.

Between the species, PI and PG had a similar growth, $p>0.05$, and SS, grew significantly more than PI and PG ($p<0.05$) (Figure 13).

2.4. Discussion

2.4.1. Analysis of mixed biofilms formed on etched and anodized Ti samples

The ability of mixed cultures to form biofilm on etched and anodized Ti surfaces was determined by crystal violet absorbance measurements (at 570 nm), enumeration of the number of viable colony-forming units (CFU) and SEM observation. The extent of colonization varied according to surface topography (Figure 5 and 6). Higher porosity samples (anodized) presented significantly ($p > 0.05$) (Figure 6) higher biofilm biomass in comparison with biofilm formed on etched samples. This happened for all conditions, control biofilm, biofilm with fluoride and biofilm with *S. salivarius*. This might be related to the existence of a superior colonization area on anodized samples, provided by their porous surface morphology. These results may imply that the development of biofilms is influenced by surface topography, in agreement with several studies (90) (91) (76). This also suggests that cells might be better retained on a surface with high lumpiness and porosity leading to a higher density of biofilms. However, much controversy still exists as to the optimal topography for implant surfaces regarding both osseointegration and antimicrobial kinetics (106) (107) (108). So, it is necessary to find equilibrium in the relation between the topography of implants and the ability to biofilm formation in order to allow a good osseointegration and implant maintenance.

In order to determine the quantity of viable cells, in the different samples and for each condition, the CFU were quantified for *P. intermedia* (PI), *P. gingivalis* (PG), *F. nucleatum* (FN) (Figure 7). For the Control biofilm essay it is possible to observe that PI did not grow of the (Figure 8) and PG and FN were both detected, although less amounts of PG when comparing to FN,. It is believed that the high growth of FN and PG around the implants and the almost absence of PI can be related to the fact that FN and PG usually form co-aggregates that can avoid the growth of PI. This “partnership”/ relation, between FN and PG, is normally found in the construction of dental plaque. The interaction among dental plaque bacteria is highly specific and primary colonizers can interact and connect with each other and with secondary colonizers. In this specific case PG is a secondary colonizer and can connect with primary colonizers. One of these primary colonizers is the Fusobacterium nucleatum that is proposed to be a bridge organism because it can bond with both primary and secondary colonizers (51). This indicates that FN was capable of adhering to the Ti surface and maybe established a “partnership” with PG. With the formation of this alliance it is possible that Pi can not adhere in a high extension due to the influence of this FN -PG complex. FN

and PG may have competed with PI by occupying a particular site or obtaining specific nutrients. In this case the interaction between the two microbial species (FN and PG) had a greater effect than the sum of the effect of both species taken individually. Moreover the colonies of PI were observed during the experiment (Figure 7), so maybe PI was able to grow in reduced numbers and under the detection limit of the used method.

Finally, regarding the differences between samples (etched and anodized) the CFU determination did not show significant differences between the two types of samples (Figure 8 and 12) in opposition to the results for crystal violet (Figure 6 and 11). It was expectable that for the surface with higher porosity the number of CFUs would be bigger compared to the sample with a smooth topography (106) (108). These results may be strange but the fact is that the crystal violet method stains not only cells but essentially any material adhering to the surface of the sample (for example, matrix components). Therefore, crystal violet staining may overestimate the number of adherent bacteria. In conclusion the crystal violet assay is a convenient tool for the rapid determination of biomass but with determination of CFUs it is possible to discuss that the amount of viable cell in biofilm is similar in both samples and maybe the matrix components are different. In anodized samples the structure of the biofilm is bigger than in etched samples as it is possible see in SEM analysis.

To document the morphology of oral biofilms and the presence of bacterial species in these biofilms, researchers can use various microscopic techniques. In this work the chosen technique was Scanning Electron Microscopy (SEM), which uses a high-energy beam of electrons to scan the surfaces of samples (121) (49). The images obtained by SEM of etched and anodized samples demonstrated the different topographies (Figure 9a and 9b) that may influence the adhesion of biofilms, being the anodized sample more porous than the etched.

The SEM micrograph of Etched and Anodized samples covered with a mixed biofilm of PI, PG and FN were also analysed. It was possible to observe the existence of biomass attached to the surfaces (Figure 9c-e) as expected by the previously CV analyse (Figure 6). The amount of biofilm formed on anodized samples was bigger than for etched samples. This qualitative analyse is in accordance with the results obtained by crystal violet and in accordance with was said about the complex matrix formed in anodized samples. Once again the topography of the samples show be an important factor in biofilm formation.

This technique is a well-accepted method of documenting biofilms. However, this method requires that samples be processed through a series of arduous fixation and dehydration steps,

which are likely to result in significant artefacts (122) and this may influence the acquisition of the images. There are also some other limitations about SEM related with the impossibility to distinguish bacteria within the biofilm that in this experiment was interesting in order to confirm the CFU results.

2.4.2. Evaluation of the presence of fluoride in biofilm formation

Considering the different assays it was also possible to study the influence of the presence of fluoride on pathogens biofilm formation. The results showed that when the medium was supplemented with fluoride, the biofilm biomass was very low, indicating an almost absence of growth in a rate of inhibition of 65% and 83%, for anodized and etched samples respectively. With the determination the number of viable colony-forming units (CFU) was also possible confirm that the presence of fluoride prevented biofilm formation by the species analysed (PI, PG and FN) (Figure 10). These results were expected due to the antimicrobial characteristics of fluoride (108). The current evidence indicates that fluoride has a multitude of direct and indirect effects on bacterial cells. These include inhibitory effects of fluoride on glycolysis and transport of carbohydrates, enzyme activities, macromolecular synthesis, and polysaccharide formation and degradation. These results confirm the influence of this kind of substrate on oral colonization and on oral implants. Even low fluoride levels reduce bacterial growth, dental plaque composition but the clinical importance of the affection of plaque metabolism by fluorides is still unclear (109) (107).

2.4.3. Analysis of the effect of a probiotic bacteria on mixed biofilm formation by pathogenic bacteria

During this chapter, beside the study of biofilm formation on titanium surfaces (anodized and etched) and effect of fluoride, the influence of probiotic bacteria in growth of pathogenic bacteria was also evaluated. Probiotics can suppress the emergence of endogenous pathogens or prevent the infection with exogenous pathogens and may also protect the host through the promotion of a beneficial host response (21).

The analysis of biomass (Figure 11) for the essay with SS demonstrated that the amount of biomass is greater in the presence of SS or almost the same. Comparing the control biofilm and the biofilm with SS there were although significant differences, $p<0.05$, between the anodized

samples. In the etched samples the result was similar to the control, this means that SS lacks the ability to join in an effective manner to etched samples. It is possible that this behaviour is due to the chemical properties of the samples or due to physical properties, such as porosity. The different treatments, with different reagents can influence the adhesion processes between the surface and the bacteria. This analysis of biomass in mixed biofilms of the pathogenic bacteria was about total biomass formed in biofilms and there was the need confirm the result with the analysis of the number of viable cells (Figure 12) in other to know and understand better the players in these biofilms.

The results obtained (Figure 12) were really different compared to the results of the control (Figure 8). Curiously in the presence of SS, PI was detected, in opposition to biofilm formed in the absence of the bacteria. Moreover, FN was not detected and it is assumed that the presence of SS placed with pathogenic bacteria, influenced the growth of this bacteria. Some bacteria, as SS, are able to produce bacteriocins that are proteinaceous bactericidal substances that inhibit the growth of closely related bacterial species or strains. The competition through bacteriocin production has been documented for many oral bacteria and this event may regulate the way bacteria interact between them (44) (28). The fact is that the reason for these occurrences are still unclear but its possible that with the inhibition of the growth of FN by SS there wasn't competition or negative interactions between FN and PI and PI was able to growth. So, SS can affect the growth of the pathogenic species through different mechanisms by affecting in a negative way the vitality or growth of a pathogen, by actively limiting the capacity of pathogen to adhere to tissue surfaces, by influencing the ability of a pathogen to produce virulence factors, or by degrading them (9). Besides SS also showed that had more ability to build a biofilm than some pathogenic bacteria and this biofilm can act as a protective coating for oral tissues against oral diseases. This biofilm may keep bacterial pathogens off oral tissues by occupying a site (118). So, probiotic bacteria can provide health benefits to the host by providing nutrients and cofactors to the host, competing directly with pathogens, interacting with the pathogen virulence factor and stimulating the host immune response (119).

2.5. Conclusion

Implant complications have significant health and financial implications to both the patient and clinician. Peri-implantitis has a multifactorial etiology in which oral biofilm is a recognizable etiologic agent. It is well demonstrated that the combination of multiple pathogenic bacteria increases the risk of peri-implant diseases. The reduction of the bacterial load to a level compatible with health is an important aspect of implant therapy. With the emergence of new technologies, identification of bacteria in the oral cavity continues to improve. So, this work aims to understand better how oral bacteria grow on different titanium implants and how they interact with each other.

Regarding the results, the formation of biofilms by pathogenic bacteria constituted only (PI PG and FN) in etched and anodized Ti samples, was concluded that these two materials had different topographies, which according to our study influence biofilm formation in different ways.

The results of crystal violet and SEM analysis showed a large amount of biomass forming on the samples anodized in comparison with the etched samples. However the results, drew attention to quantification of viable cells, shows that there are an approximate number of viable cells in both samples which leads us to believe that the samples anodized support the formation of complex extracellular matrix because the cell population is relatively equal. This could be interesting for future studies, since there are other studies indicating that biofilm formation can protect the wear of the implants allowing a longer duration of these implants.

So, the anodized material can be an optimal solution for the construction of dental implants allowing a good osseointegration, a microbial attachment similar to the titanium etched materials and a complex extracellular matrix formation that could protect the implant against wear and protect of the failure of the implant.

Concerning the differences in bacterial colonization on samples, one interesting result was related with the fact that Pi did not growth and the explanation found was that FN and PG are able to form “congregate” pairings between them preventing directly the growth of PI. However PI is able to grow in that environment, but only in trace amounts maybe because of space or nutritional limitations. Thus multi species environments like oral flora are very complex to study due the numerous interactions between the bacteria.

During this chapter the influence of fluoride and probiotic bacteria (*Streptococcus salivarius*) on biofilm formation was also evaluated. The presence of fluoride showed to inhibit biofilm formation on those biomaterials with high rates of inhibition. The results came in other to confirm the greater influence of this subtract in biofilm formation. The inhibitory effects of fluoride can be

used to reduce bacterial growth but it is still necessary test the influence of this subtract on implants materials.

The great influence of probiotic bacteria, *S. salivarius* (SS) was also validated on biofilm control. It, was demonstrated that probiotic bacteria may have direct influence on the growth of pathogenic bacteria, such as FN because with the addition of SS, PI was detected and FN not, in opposition to biofilm formed in the absence of the bacteria. The inhibitory regulation of FN by SS can be related with the production of bacteriocins that are proteinaceous bactericidal substances that inhibit the growth of bacterial species or strains. Can be also considered the competition between the pathogenic bacteria and the probiotic that actively limiting the capacity of pathogen to adhere to tissue surfaces, by influencing the ability of a pathogen to produce virulence factors, or by degrading them.

Therefore, in conclusion Ti anodized samples may be a good material for the production of dental implants due to its topography, fluoride had a great inhibitory effect on biofilm formation on Ti surfaces and finally probiotic bacteria have influence in pathogenic bacterial interactions.

3. Chapter III

Considering that a carbon source can significantly affect the growth of oral bacteria, the aim was to assess the effect of a new sugar in the growth of several oral bacteria by comparison with glucose.

3.1. Introduction

The organisms in supragingival plaque are considered to be the major aetiological agents of dental caries and periodontal diseases. Some of them like *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sobrinus*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are responsible for most of these oral disease.

Current treatment regimes for plaque-related diseases involve the mechanical removal of the causative organisms, which is a painful procedure. With the recognition that each of these diseases is associated with a specific organism, or group of organisms, increasing interest is emerging in the use of antimicrobial agents to supplement these rather crude mechanical procedures (123). However the overuse of antimicrobial drugs has been responsible for bacterial resistance and so the use of these drugs is not anymore an interesting solution. Therefore, the concept of probiotic and prebiotic therapy has been considered with extremely curiosity for improve of oral health (20).

The interest in probiotics, prebiotic and the modulation of the microbiota for restoring and maintaining health have earned a lot of attention over the past decade (29). Prebiotics were first defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria and thus improves host health. However, a prebiotic effect has been attributed to many food components, sometimes without consideration to the criteria required (124).

The use of probiotics has potential, but still requiring the publication of further well-designed, randomized, controlled trials (especially in caries and periodontal disease) before they could be recommended as prophylaxis for caries or periodontal disease prevention. So, the direct application of probiotics is still a risk but there are other solutions that can be studied. The use of the commensal bacteria is one of them. Probiotics, perhaps in combination with prebiotics or other substances, may become an important mean towards preventing and treating the disease. This means that prebiotics or natural substances that stimulates selectively the growth and/or activity of bacteria associated with health and wellbeing can be one big approach (125).

The concerning of selective stimulation of growth and/or activity of bacteria, is the most contentious and difficult to do. Indeed, it requires more studies to know which substances have direct effects in the growth of probiotic bacteria. This study, may represent only the “tip of the iceberg” because the potential benefits of prebiotic therapy promise to be almost limitless (125).

Thus, the main objective of this chapter is to determine the influence of a new different sugar in the growth of diverse pathogenic and probiotic bacteria. Henceforth, if these sugars have different influences on the growth of these bacteria these factors can be used to favour the growth of the ones, which are beneficial to oral health, namely probiotic bacteria.

3.2. Materials and Methods

For this study six different oral bacteria were used, namely, *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sobrinus* (ATCC 20627), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), *Prevotella intermedia* (ATCC 25611) and *Fusobacterium nucleatum* (ATCC 10953). Adsitionally, one probiotic, *Streptococcus salivarius* (ATCC 7073), was also used. These bacteria were grown on blood agar plates (Boold Agar Base II, Oxoid, Basigstoke, UK) supplemented with 5% horse blood (Biotrading, Keerbern, Belgium). *S. mutans, S. mitis, S. sobrinus*, *A. actinomycetemcomitans* and *S. salivarius* were grown at 37°C in an atmosphere with 5% of CO_2. While *P. intermedia* and *F. nucleatum* grown under anaerobic conditions in anaerobic jars containing 80% N_2, 10% CO_2 and 10% H_2 (Anoxomat, the Netherlands) and incubated at 37°C.

The day before the start of each experiment, bacteria were collected from the blood agar plates and were grown overnight in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, USA).

3.2.1. Growth curves

After the inoculation and incubation of the bacteria as previously mentioned, cells were harvested by centrifugation at 7500 rpm during 10 min and were resuspended in 10 mL of fresh BHI medium. The optical density of each suspension was measured at 600 nm using a spectrophotometer. The suspensions were diluted in distilled water to achieve a cell density of $1x10^7$ colony forming units (CFU)/mL.

Six different conditions were tested in this experiment:

1. Negative Control without bacteria (BHI medium only) - Control (-)
2. Positive control (bacteria suspension $1x10^7$ (CFU)/mL in BHI) - Control (+)
3. Bacterial suspension and Glucose (10 mg/mL) - Gluc/10
4. Bacterial suspension and Glucose (50 mg/mL) - Gluc/50
5. Bacterial suspension and C7 (10 mg/mL) - C7/10
6. Bacterial suspension and C7 (50 mg/mL) - C7/50.

Bacterial growth was monitored spectophotometrically, with a Thermo Ascent ELISA reader, at 600 nm until, the stationary phase was reached. To assess the effect of glucose and C7 on the growth of the different bacteria, bacterial suspensions were monitored during 10h with measurements every 30min.

For *S. mutans, S. mitis, S. sobrinus*, *A. actinomycetemcomitans* and *S.* salivarius, the assays were performed in 96 well plates and for anaerobic bacteria *P. intermedia*, *F. nucleatum,* the experiments were performed in 24 well plates with the same concentration and the same conditions as previously mentioned. The differences were the volume in each well and the absorbance was read at 0h, 24h and 48h with a Thermo Ascent ELISA reader, at 600 nm.

All experiments were performed in triplicate.

3.3. Results and discussion

In this part of the study the influence of one energy compound in the growth of both pathogenic and probiotic bacteria was investigated. In this way it was possible to identify pathways to direct the growth of the pathogenic as well as beneficial bacteria. The identification of additional compounds that will enhance the growth of probiotic organisms (eg, the development of more effective and safer prebiotics and selection) is one of the main goals in the future.

In order to study the growth of both pathogenic and probiotics bacteria, six different conditions were tested in this experiment. The bacteria *A. actinomycetemcomitans* (Figure 13), *S. mutans, S. mitis, S. sobrinus* (Figure 14) and the probiotic *S. salivarius* (Figure 16) were incubate in aerobic conditions and the other pathogenic, *P. intermedia* and *F. nucleatum* (Figure 15) in anaerobic conditions.

Comparing all the bacteria Control(+) the growth was more a less the same for the bacteria including to the probiotic bacteria. The only exception was for *A. actinomycetemcomitans* (Figure 13), this bacteria grew less than the orders.

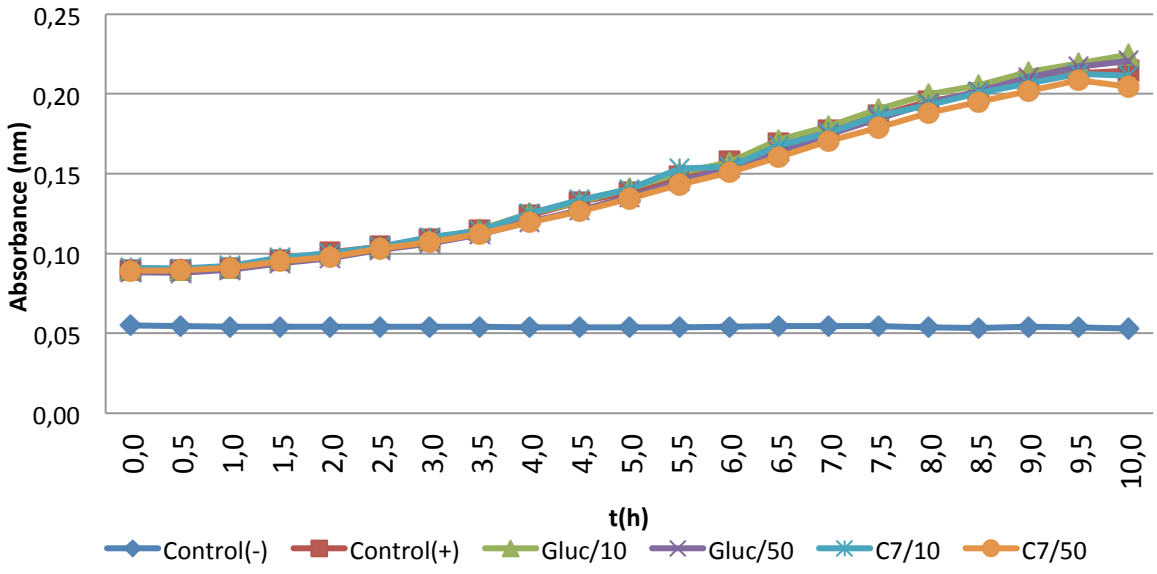

Figure 13. Growth curve of *A. actinomycetemcomitans for six conditions* based on different energy sources (BHI medium supplemented with glucose or the sugar under study - C7).

In regards to *A. actinomycetemcomitans,* it was difficult (Figure 13) to distinguish the *"plateau"* corresponding to the stationary phase because this bacteria needs more that 10h to get on stationary phase. Moreover for this species there were no differences on growth under the several conditions assayed.

For the other bacteria, *S. mutans, S. mitis* and *S. Sobrinus,* it was possible to observe differences in the growth caused by the different energy sources and their different concentrations (Figure 14).

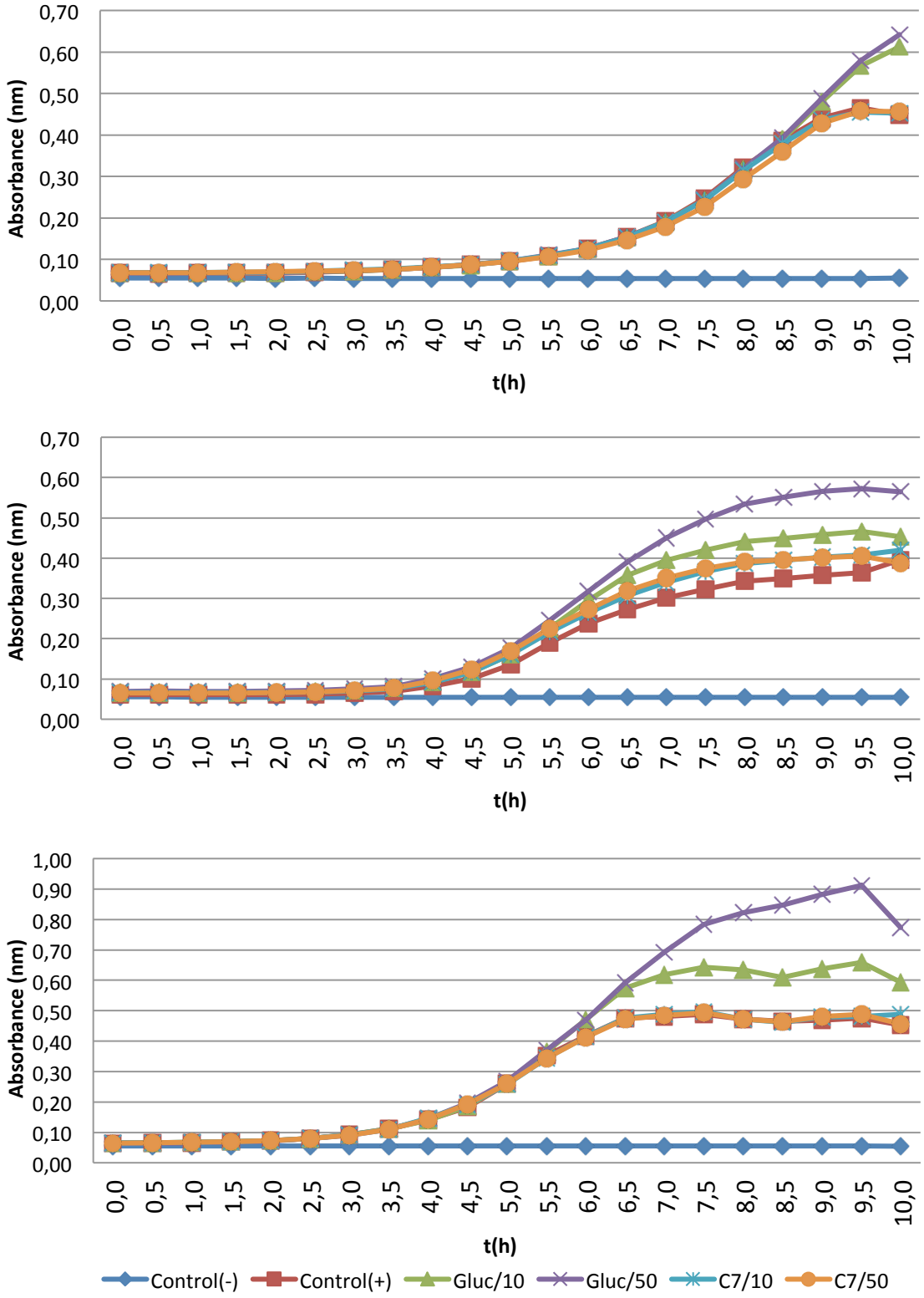

Figure 14. Growth curve of *S. mitis, S. mutans* and *S. sobrinus*, respectively, *for six conditions* based on different energy sources (BHI medium supplemented with glucose or the sugar under study - C7).

In the Figure 14 it is possible to observe that the presence Glucose and C7 have different influences in the growth of these bacteria. For the three bacteria (*S. mitis, S. mutans* and *S. sobrinus*) the growth was improved in the presence of glucose compared to the control and the C7.

With regard to the different concentrations used it was noted that for *S. mitis* (Figure 14) the growth was similar, either for C7 or glucose with the same concentration. For *S. mutans* and *S. sobrinus* the growth was improved in the presence of the higher concentration of glucose (Gluc/50).

According to the literature, the availability of suitable nutrition is a prime environmental factor regulating selection, establishment and survival of microorganisms (126) (127) (128).

The second part of this experiment looked at the influence of different energy sources but on anaerobic oral bacteria, *P. intermedia* and *F. Nucleatum* (Figure 15).

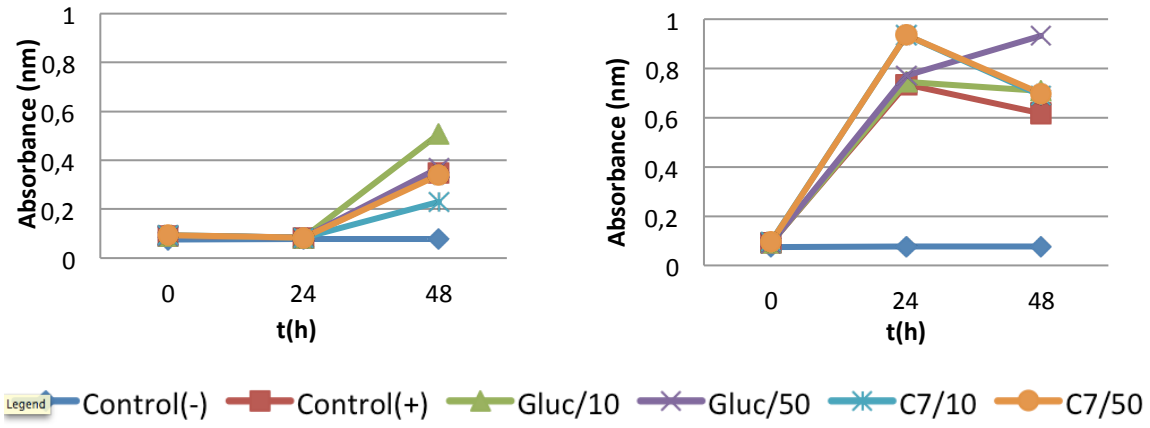

Figure 15. Growth curve of *P. intermedia* (left) and *F. nucleatum* (right), *for six conditions* based on different energy sources.

The results show that these bacteria grow slower than the aerobic bacteria studied and the influence of the energy sources is a little different but the conclusion is very similar (Figure 15). For *P. intermedia*, it was possible to notice differences in the growth caused by different energy sources, the growth was greater in the presence of Glucose (10 mg/mL) and for *F. nucleatum* the growth was greater in the presence of glucose (50 mg/mL) compared to C7. The differences between the concentrations for *P. intermedia* were different from expected because the conditions with lower concentrations grew more than the highest. For *F. nucleatum* the conditions with C7 grew in same way and with glucose the Gluc/50 grew more than Gluc/10.

The results obtained in this part of the work showed the principal differences between aerobic and anaerobic bacteria, which are mostly related with the growth time. Regarding the sources of energy as aerobic pathogenic bacteria these anaerobic pathogenic bacteria had higher growth in the presence of Glucose. Once again the influence of different sources of energy proved be determinant for they growth. According with the bibliography, availability of suitable nutrition is a prime environmental factor regulating selection, establishment and survival of microorganisms (126) (127) (128). So, is in this context the study of growth of pathogenic oral bacteria and probiotic is very interesting.

For the probiotic bacteria, *S. salivarius*, there were also differences between the conditions and in presence of Glucose and C7 *S. salivarius* grew more than in the absence of those sugars. In this specific case it is possible see differences between the condition more because the concentrations than for the energy sources. In the Figure 16 it is possible observe that for higher concentrations of sugar (Gluc50 and C7/50) the bacteria grew more than for the lowers (Gluc10/ and C7/10).

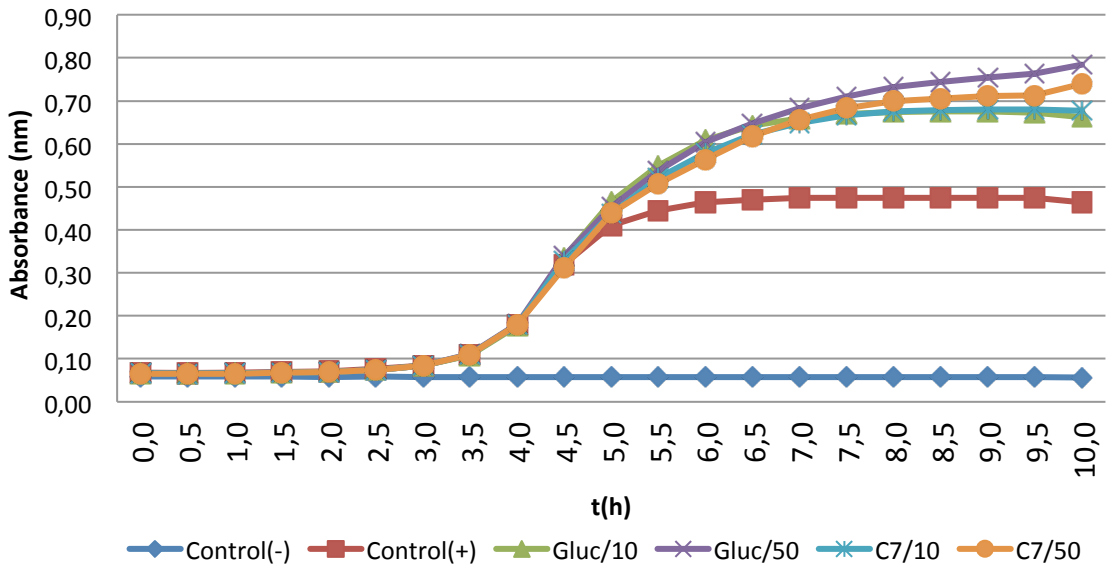

Figure 16. Growth curve of *S. salivarius for six conditions* based on different energy sources (BHI medium supplemented with glucose or the sugar under study - C7).

According to the results it is possible conclude that the behaviour of pathogenic bacteria is different from the behaviour of probiotics. The probiotic bacteria in presence of C7 grow as much as with glucose while the pathogenic bacteria grew more always with glucose, regardless of

concentration. So, these results showed that there were influences of the energy sources and in some cases concentrations influence also.

It was expected differences in the growth for different concentrations and also for different sources of energy. However the specific influences were unknown, the previous studies never compered before the growth in the presence of Glucose and this sugar, C7. Due this fact this study is important to a better understanding of nutritional influences on pathogenic oral bacteria and on probiotic bacteria, as will be discuss follows.

3.4. Conclusion

In the field of periodontal healthcare, probiotics might provide opportunities to prevent plaque-related periodontal inflammation. Moreover, the presence of pathogenic species and the increase of some bacteria called ''beneficial bacteria'' (probiotic bacteria) can prevent some of the most common oral diseases.

Probiotics in combination with prebiotics or other substances, become an important means towards preventing and treating the disease. The main idea was to use a sugar that stimulates selectively the growth and/or activity of bacteria with a similar prebiotic behaviour.

The energy sources tested were glucose and C7 at different concentrations and the results showed that in generality pathogenic bacteria grew more in the presence of glucose than in presence of C7. While, the probiotic bacteria grow in a similar way for both sources of energy. So, the the results showed that pathogenic bacteria are not stimulated by C7, having more a less the same growth as control.

The results of this chapter may contribute to the study of a new therapy linked to the use of different sources of energy to different metabolisms for different bacteria. This way the sugar can be used to favour the growth of the probiotics, which are beneficial to oral health. So, the use of these sugar allied with probiotics may be a start of a development of a new therapy.

However there are some limitations on the use of this kind of therapies concerning of selective stimulation of growth and/or activity of bacteria, is the most contentious and difficult to in multi species environments. More studies are necessary to understand the mechanisms behind the metabolism of pathogenic and probiotic bacteria. This practice, however, represent only a small part of knowledge in this area because the potential benefits of probiotic therapy promise are almost limitless.

4. Chapter IV

Conclusions and Future perspectives

This chapter presents the conclusions obtained during the experimental work and also some suggestions for future work.

4.1. Conclusions and future perspectives

The improvement of oral health has always been an important subject of interest in our society. Considering that oral health problems affect a large part of the population the necessity to develop more effective mechanisms to combat periodontal diseases becomes of major importance.

Despite the numerous developments in oral products and technologies, there is still a need to know and to understand how these bacterial diseases affect the general health

With the advance in new materials and treatments for the improvement of oral health the requirements arising from society have changed. One of these changes is denoted in the exponential increasing usage of dental implants. However, several complications are still associated with oral implants and have significant health and financial implications.

This project aimed to study biofilm formation on titanium surfaces (anodized and etched) and to evaluate the effect of fluoride and probiotic bacteria on biofilm formation. Furthermore, it was also a goal to study the influence of different sugars (Glucose and C7) on the growth of pathogenic and probiotic oral bacteria.

When adhered to titanium surfaces, *Fusobacterium nucleatum* (FN) and *Porphyromonas gingivalis* (PG) may form co-aggregates avoiding the adhesion of *Prevotella intermedia* (PI). Moreover, probiotic bacteria, *Streptococcus salivarius* (SS), may have a direct influence on the growth of pathogenic bacteria, such as FN. However, these interactions are still unclear and there is a need to study these in greater detail.

The presence of fluoride showed to have a negative influence on pathogens' biofilm formation. These effects of fluoride were noticed by a decrease in biofilm biomass as well as a decrease of viable cells in the biofilm. These results were, in part, expected due to the antimicrobial characteristics of fluoride, which has direct and indirect effects on bacterial cells including inhibitory effects on glycolysis and transport of carbohydrates, enzyme activities, macromolecular synthesis, and polysaccharide formation and degradation.

Regarding the different titanium surfaces, the formation of biofilms on anodized samples resulted in a higher biomass than on the etched samples. However the number of biofilm viable cells was similar in both samples, which may indicate that biofilms formed on anodized samples possess more extracellular matrix. The interest in anodized samples grows with these results, because the anodized material is an optimal solution for the construction of dental implants allowing a good osseointegration, a microbial attachment similar to the titanium etched materials and a complex extracellular matrix formation that could protect the implant against wear.

Despite these results it also important to control the presence of pathogenic species in oral environment, so another important part of this study addresses the usage of probiotic bacteria to improve oral health.

The combination of probiotics with other substances can become an important means towards preventing and treating the disease. The different sugars assayed, C7 and glucose, showed different influences on growth of pathogenic and probiotic bacteria. The sugar C7 favoured only the growth of the beneficial oral bacteria. Henceforth, the use of these sugar allied with probiotics may be a start of the development of a new therapy. However there are some limitations on the usage of this kind of therapies concerning the selective stimulation of growth and/or activity of bacteria, as it is the rather contentious and difficult to perform in multi species environments. More studies are necessary in other to understand the mechanisms behind the metabolism of pathogenic and probiotic bacteria. The exact nature of pathogenic and probiotic interactions needs further investigation related with competition, production of inhibitory substances and environmental conditioning. With the knowledge of these interactions it could be possible to implement new oral therapies using selected probiotic bacteria or substances that can work as prebiotics having beneficial influences on oral health.

In conclusion several studies have already been performed on the microbial colonization of surfaces, however the study of these two types of treatment samples and the influence of probiotic bacteria remains unknown. Therefore, this study permitted to increase and to expand towards new perspectives the general knowledge about oral health in specific in biofilm formation, oral implants and probiotics.

As future work, it would be interesting to perform an analysis with further precision of the total amount of bacteria on biofilms and compare the results with the obtained in this work as the analysis of direct inhibition not only of on PI PG and FN but also in other bacteria. The execution of a QPCR analysis would permit the quantification of the bacteria and confirm the results obtained by CFU analysis. It was proved during this work that there are specific interactions between these bacteria. However the oral environment is a multispecies community composed by many other bacteria and the interactions between them are very complex. So, interpretations of these interactions assessed directly between 3 or 4 species (PI, PG, FN and SS) should take into account that their effect may not be detected in the behaviour of the species within a community.

Therefore biofilm formation on oral implants surface is one of the main causes for implant failure and this may be the cause for peri-implant tissues inflammation (peri-implantitis). The

present work tried to understand how the bacteria react to implant materials and concluded that Ti anodized can be a better solution than Ti etched. However, there is still the need to make more reliable tests concerning biomass formation in the samples. One possibility is the identification of the congregating complexes between the oral bacteria using a staining technique or a technique that allows us to distinguish the different bacteria and the extracellular matrix material. This should help to understand better the interactions between the bacteria and the different amount of biomass achieved to the surfaces.

The two Ti samples used with different treatments that were used could be also tested “in vivo” in other to remain in an environment more similar to the one where the samples need to be placed. It is very difficult achieve oral cavity similar conditions. Thus for the future, an “in vivo” study would be very important for the development of a clinical trial of these two materials adapted for oral implant industry.

Regarding the use of different energy sources to induce different growth on bacteria there is still a lot to do to understand to use these as a therapy. During this work only some of the bacteria present in an oral environment were used and the essays were preformed for each bacterium. The fact is that in oral cavities there are hundreds of different types of bacteria and they have a close relationship so, it would be interesting to use other pathogenic bacteria and create a multi-species environment in order to better understand the interactions between bacteria and their metabolisms and consequently the influence of energy source. It is still necessary to further understand the direct relationship between the energy source, c7 and SS studying the components present in the cultures.

These are some of the many things that could be done in this immensely vast field. Increasingly, the world's population is more focused on oral health that in the past and that is the most important driving force under the research in this area.

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